Techniques and applications of in vitro orchid seed germination

Chapter · January 2008

CITATIONS
32

READS
11,192

6 authors, including:

Scott Stewart
Millennium Park Foundation
36 PUBLICATIONS **780** CITATIONS
SEE PROFILE

Michael E. Kane
University of Florida
87 PUBLICATIONS **1,386** CITATIONS
SEE PROFILE

Wagner A. Vendrame
University of Florida
117 PUBLICATIONS **851** CITATIONS
SEE PROFILE

Techniques and Applications of *In Vitro* Orchid Seed Germination

Philip Joseph Kauth[1*] • Daniela Dutra[1] • Timothy Robert Johnson[1] • Scott Lynn Stewart[2] • Michael E. Kane[1] • Wagner Vendrame[3]

[1] Department of Environmental Horticulture, University of Florida, PO Box 110675, Gainesville, FL 32611, USA
[2] *Phyto*Technology Laboratories, 14335 West 97th Terrace, Lenexa, KS 66215, USA
[3] Tropical Research and Education Center, University of Florida, 18905 SW 280th Street, Homestead, FL 33031-3314, USA
Corresponding author: * **pkauth@ufl.edu**

Keywords: asymbiotic, cryopreservation, dormancy, mycobiont, mycorrhiza, symbiotic

ABSTRACT

In nature orchid seeds germinate only following infection by mycorrhizal fungi that provide the developing embryo with water, carbohydrates, minerals, and vitamins. Orchid seeds were first germinated at the base of wild-collected potted orchids, but germination was unreliable and seedling mortality rates were high. *In vitro* germination techniques, which were developed in the early 1900s, have resulted in more reliable germination and propagation of many orchid taxa. The earliest *in vitro* orchid seed germination techniques utilized mycorrhizal fungi found in nature to stimulate germination and seedling development. In 1922 Lewis Knudson germinated orchid seeds *in vitro* by sowing seeds on sterile nutrient medium amended with sucrose. This technique is known as asymbiotic seed germination since no fungal mycobiont is used to promote germination. For both symbiotic and asymbiotic orchid seed germination to be effective, many conditions must be addressed such as photoperiod, temperature, and mineral nutrition. In the case of symbiotic germination, another important factor is fungal compatibility. In recent years, the limitations that seed dormancy poses to the germination of orchid seeds have also been examined. In this chapter techniques and applications of asymbiotic and symbiotic orchid seed germination will be discussed in relation to photoperiod, temperature, nutrition, seed dormancy, and fungal mycobionts.

1. INTRODUCTION

Perhaps no plant family is as intriguing and complex as the Orchidaceae. The Orchidaceae is the largest family of plants with 17,000-35,000 species (Dressler 1993). Members of this family are found on every continent except Antarctica, with the highest diversity in tropical regions of Southeast Asia, South America, and Central America. Seventy percent of all orchid species are epiphytes, but terrestrial, aquatic, and lithophytic species can also be found (Dressler 1993).

A defining characteristic of orchids is their seeds, which are adapted for wind dispersal. Orchid seeds are incredibly small and contain an undifferentiated embryo that lacks enzymes to metabolize polysaccharides (Manning and van Staden 1987; Molvray and Kores 1995). The testa (seed coat) of orchid seeds is often hard, yet thin (Molvray and Kores 1995). Sugars are present in orchid embryos in the form of sucrose, fructose, maltose, rhamnose, and glucose, but these sugars are either utilized fully prior to germination or are present in insufficient quantities to support and sustain germination (Manning and van Staden 1987). Although seeds utilize lipids and proteins as the major nutrient source, embryos also lack enzymes to convert lipids to soluble sugars (Manning and van Staden 1987). Since orchid seeds can not metabolize polysaccharides and lipid, they utilize a mycorrhizal relationship with compatible fungi (=mycobiont) during germination and early development (Rasmussen *et al*. 1990a). Following penetration of the embryo, mycobionts provide embryos with water, carbohydrates, minerals, and vitamins (Rasmussen 1992; Yoder *et al*. 2000). Mycobionts are important for the initiation of seed germination by stimulating glucose and enzyme production, reserve mobilization, and post-germination nutrient support (Manning and van Staden 1987).

In the past 15 years, great advances have been made in the effectiveness of *in vitro* orchid seed germination. In this chapter, we provide a brief history of orchid seed germination followed by discussion of methods, techniques, and current issues regarding both asymbiotic and symbiotic germination. Although several important older articles are discussed, the majority of cited articles have been published in the past 15 years.

Abbreviations: 2-iP, 6-(α,α-dimethylallylamino)-purine; **ABA**, abscisic acid; **BA**, benzyladenine; **CMA**, corn meal agar; **DAP**, days after pollination; **GA**, gibberellic acid; **K**, kinetin; **LN**, liquid nitrogen; **NAA**, naphthalene acetic acid; **OMA**, oat meal agar; **PDA**, potato dextrose agar; **PGR**, plant growth regulator; **TDZ**, thidiazuron; **TZ**, tetrazolium; **WAP**, weeks after pollination; **Z**, zeatin

2. THE ORCHID SEED

The species diversity in the Orchidaceae can also be seen in the diverse shapes, sizes, and patterns of the seeds as well. Orchid seeds are very minute and range in length from 0.05 to 6 mm, 0.01 to 0.93 mm in width, and weigh 0.3 to 14 µg (Arditti, 1967; Arditti and Ghani 2000). Seed capsules may hold anywhere between 1,300 to 4 million seeds (Arditti 1967). Shapes are also various including filiform, fusiform, clavate, and ellipsoidal seeds (Molvray and Kores 1995).

Orchid seeds share a common characteristic of a reduced embryo and the absence of endosperm (Prutsch *et al.* 2000), with the exception of *Sobralia* and *Bletilla* seeds that have a rudimentary cotyledon (Arditti 1967). These characteristics lead to various surface depressions and sculpturing patterns in the testa, which in turn increase the resistance to air and allow seeds to remain air-borne or floating on water for long periods (Prutsch *et al.* 2000). The testa is normally derived from the outer integument, but as in the case of *Paphiopedilum delenatii* the testa is derived from both the inner and outer integument (Molvray and Kores 1995; Lee *et al.* 2006). In most species the testa is usually only one cell thick, but made up of 20 to 600 cells (Molvray and Kores 1995; Prutsch *et al.* 2000).

The embryo is attached to the testa by several strands or cells, contains dense cytoplasm, and consists of as few as ten cells (Stoutamire 1964). At early globular stages, plastids with starch are visible, but soon disappear during the mature globular stage (Lee *et al.* 2006). Durng the mature globular stage, the starch plastids are replaced by lipid and protein bodies (Lee *et al.* 2006). Cuticular substances appear in the surface wall cells of the embryo during the early globular stage, but are free from the suspensor region (Lee *et al*. 2006). The suspensor serves as a channel for free movement of nutrients and water as well as a food storage site for the embryo (Yeung *et al.* 1996). The two-cell thick inner integument (carapace) dehydrates and compresses around the embryo at full maturity (Lee *et al*. 2005). A layer inside the inner integument becomes cutinized and a layer outside the inner integument becomes lignified at seed maturity (Yamazaki and Miyoshi 2006). The lignification and cutinization may serve to strengthen the carapace, while the tight fitting carapace may inhibit embryo growth by restricting growth mechanically or chemically (Yamazaki and Miyoshi 2006).

3. SEED GERMINATION HISTORY

Interest in orchid seed germination began in the 1800s. Early attempts to initiate germination involved placing seeds onto organic substances such as sphagnum moss, bark, or leaf mold, but this often proved unsuccessful (Arditti 1967). Another method utilized by early growers was to germinate seeds in pots with the wild-collected mother plants. Bernard and Burgeff were the first to recognize the role of fungi in orchid seed germination by co-culturing fungi with orchid seeds (=symbiotic germination) (Bernard 1899; Burgeff 1909). They experimented with symbiotic seed germination, which is the co-culture of fungi with orchid seeds. Although seeds did not germinate readily, they concluded that orchid seeds could germinate *in vitro* in the presence of an appropriate mycorrhizal fungus (Knudson 1922). Bernard, however, did germinate seeds of *Cattleya* and *Laelia* in the absence of a fungus by placing seeds on salep, a powder obtained from tubers of *Ophrys* (Knudson 1922; Arditti 1967).

Based on initial experiments by Bernard and Burgeff, Lewis Knudson further examined orchid seed germination. Using nutrient solutions supplemented with 1% sucrose, Knudson (1922) successfully germinated seeds of several epiphytic orchid genera. From these initial experiments Knudson demonstrated that orchid seeds could germinate *in vitro* without a mycorrhizal fungus (=asymbiotic germination). To germinate seeds that did not readily germinate in his early studies, Knudson developed solution C, which is widely used as Knudson C Medium (Knudson 1946).

Asymbiotic germination represents an ideal system for studying the growth and development of orchid seeds and seedlings. Although the first asymbiotic seed germination experiments focused on tropical orchids, research in the past 20 years has grown to include terrestrial species. While asymbiotic germination is often a more popular technique for orchid seed germination, symbiotic seed germination has recently gained popularity for conservation and restoration projects. Factors such as photoperiod, temperature, and culture media, as well as seed dormancy may influence rates of both asymbiotic and symbiotic germination. More recent research has not only examined germination, but the subsequent growth and development of protocorms and seedlings (**Fig. 1**).

4. ASYMBIOTIC MINERAL NUTRITION

Since Knudson demonstrated the feasibility of asymbiotic germination, the role of mineral nutrition in tropical orchid seed germination has been researched extensively. Many different culture media have been developed since Knudson's original formula was published (see **Table 1** for examples). Although many of these media have only minor differences in composition, growth and development of species may be significantly affected. The majority of research on mineral nutrition requirements of orchid seeds was conducted prior to 1970. The roles nitrogen form and concentration, carbohydrate source, vitamins, and plant growth regulators (PGRs) play in asymbiotic germination were examined in earlier studies. More recently the role of individual media components have not been as extensively investigated, but rather commercially prepared media are often used to conduct screens to obtain satisfactory germination. Such studies also focused on characterizing the growth and development of protocorms and seedlings to more precisely track growth rates (**Table 2**).

4.1. Nitrogen

Nitrogen has long been considered as an important role in the germination of orchid seeds. Recent reports have shown that while one asymbiotic culture media may support initial germination, another medium may better support subsequent development. Stenberg and Kane (1998) and Kauth *et al*. (2006) reported high seed germination of *Encyclia boothiana* var. *erythronioides* and *Calopogon tuberosus*, respectively, on Knudson C (Knudson 1946). The high germination percentages on Knudson C were attributed to high ammonium content, which can be utilized by seeds during early germination and development (Stenberg and Kane 1998; Kauth *et al*. 2006). Seedling fresh weight of *Cattleya* and *Cymbidium* hybrids was greater when grown on a medium with a high ratio of ammonium to nitrate (Curtis and Spoerl 1948).

While Knudson C also promoted seedling development of *E. boothiana* (Stenberg and Kane 1998), *C. tuberosus* seedlings developed to more advanced stages on P723 Orchid Seed Sowing Medium (*Phyto*Technology Laboratories, Inc., Shawnee Mission, KS) rather than on Knudson C (Kauth *et al*. 2006). The limited development of *C. tuberosus* on Knudson C was attributed to a high nitrate concentration and the inability of the protocorms to utilize nitrates during early growth and development (Raghavan and Torrey 1964). Peptone, an organic nitrogen source present in P723, may have contributed to the increased seedling development by supplying auxin-like compounds or various amino acids (Curtis 1947; Kauth *et al*. 2006). However peptone responses may be species specific. Seed germination percentages of *Paphiopedilum insigne* and *P. hirsutissimum* were approximately 30% higher with peptone than without peptone (Curtis 1947). Adversely, seed germination of *Phaius grandiflorus* and *Habenaria clavellata* (renamed to *Platanthera clavellata*) was hindered in the presence of peptone. Increased uniformity of seedling development was also observed in seedlings cultured in the presence of peptone (Curtis 1947).

Although ammonium was found beneficial in asymbiotic germination of *E. boothiana* var. *erythronioides* and *C. tuberosus*, seed germination of other terrestrial orchids may be inhibited by it. Germination and growth of *Dactylorhiza incarnata* seeds, a European terrestrial orchid, were reduced in the presence of ammonium (Dijk and Eck 1995b). Dijk and Eck (1995a) also found that as nitrogen concentration increased, protocorm weight decreased in two species of *Dactylorhiza*. Likewise, a high ratio of ammonium to nitrate reduced the germination of *Vanda tricolor* (Curtis and Spoerl 1948).

Amino acids have also been used as a substitute nitrogen source. Raghavan (1964) reported that only certain amino acids increase seed germination of *Cattleya*. Glycine, the simplest amino acid, decreased overall germination of *Cattleya* seeds from 53% to 41%. However, germination in the presence of arginine, proline, and glutamine was similar to that with ammonium nitrate (Raghavan 1964). Spoerl and Curtis (1948) also reported that glycine significantly reduced germination of *Cattleya* seeds after 2 months when compared with other amino acids. However, after 5 months germination in the presence of glycine increased from 22.5% to 64%. Amino acid enzyme systems within developing embryos change over time. Amino acids may not be available as initial nitrogen sources, but may be metabolized after a certain period of time (Spoerl and Curtis 1948). Various orchid species respond differently to various amino acids during germination, and therefore further investigation should be carried out. Since not all amino acids are beneficial for seed germination, combinations of amino acids may increase germination (Spoerl and Curtis 1948).

Table 1 Comparative mineral salt content of commonly used asymbiotic orchid seed germination media: Knudson C (KC), Malmgren Modified Terrestrial Orchid Medium (MM), *Phyto*Technology Orchid Seed Sowing Medium (P723), Murashige and Skoog (MS), Vacin and Went (VW). Formulations based on those provided by *Phyto*Technology Laboratories, LLC.

	KC	MM	P723	½MS	VW
Macronutrients (mM)					
Ammonium	13.82		5.15	10.31	7.57
Calcium	2.12	0.24	0.75	1.50	1.93
Chlorine	3.35		1.50	1.50	
Magnesium	1.01	0.81	0.62	0.75	1.01
Nitrate	10.49		9.85	19.70	5.19
Potassium	5.19	0.55	5.01	10.02	7.03
Phosphate	1.84	0.71	0.31	0.63	3.13
Sulfate	4.91	0.92	0.71	0.86	4.92
Sodium		0.20	0.10	1.51	0.20
Micronutrients (µM)					
Boron			30	50	
Cobalt			0.03	0.11	
Copper			0.03	0.10	
Iron	90	100	50	50	100
Iodine			1.20	2.50	
Manganese	30	10	30	37.90	30
Molybdenum			26	0.52	
Zinc			9.20	30.00	
Undefined Organics (mg/l)					
Biotin		0.05			
Casein hybrolysate		400			
Folic acid		0.5			
Glycine		2.0			
Myo-inositol		100	100		
Nicotonic acid			1.0		
Peptone			2000		
Pyridoxine			1.0		
Thiamine			10		
Total N (mM)	24.31	n/a	unknown	30.01	12.76
NH_4:NO_3	1.32	n/a	0.52	0.52	1.46

Table 2 Developmental stages of orchid seed germination (from Stenberg and Kane 1998; Stewart and Zettler 2002).

Stage	Description
0	No germination, viable embryo
1	Imbibed embryo, still covered by testa (=germination)
2	Embryo enlargement, testa rupture
3	Appearance of protomeristem
4	Elongation of protomersitem; emergence of first true leaf
5	Elongation of first true leaf
6	Appearance of second leaf

Edamin, a lactalbumin hydrolysate with peptides and 18 amino acids, increased the germination of a *Cattleya* × *Laelia* hybrid (Ziegler *et al.* 1967). On the media with Edamin, embryos became green faster and seedling dry weight was greater than seedlings cultured on media without Edamin. Tissue analysis of seedlings cultured on Edamin yielded increased levels of amino acids. Glutamine, asparagine, and gamma amino butyric acid were detected in seedling tissue, but these amino acids were not found in Edamin. Complex organic nitrogen sources such as Edamin might be used an amino acid building component (Ziegler *et al*. 1967).

Majerowicz *et al*. (2000) reported increased growth of *Catasetum fimbriatum* seedlings in the presence of the amino acid glutamine, over media containing ammonium or nitrate. Stewart and Kane (2006a) reported improved germination and subsequent development of *Habenaria macroceratitis* on Malmgren Modified Terrestrial Orchid Medium (Malmgren 1996). Malmgren Modified Terrestrial Orchid Medium as prepared by *Phyto*Technology Laboratories, Inc. is a low mineral salt medium with glycine as the sole nitrogen source. Researchers have suggested that nitrogen in the form of amino acids may be more readily available to germinating seeds or developing protocorms than inorganic nitrogen sources (van Waes and Debergh 1986b; Malmgren 1993; Anderson 1996; Malmgren 1996; Stewart and Kane 2006a). When inorganic nitrogen, such as ammonium, is utilized by germinating seeds, the nitrogen is converted to amino acids (Majerowicz *et al*. 2000). Using amino acids as the sole nitrogen source in orchid seed germination may lead to more efficient nitrogen assimilation by bypassing certain nitrogen conversion steps; however, this may be species specific and should be further investigated.

4.2. Carbohydrates

The role of carbohydrates in orchid seed germination has long been studied. Since orchid seeds have minimal carbohydrate reserves, an

exogenous source of carbohydrate is required for *in vitro* orchid seed germination. Two sources of carbohydrates are available to the germinating embryo during the first stages of development in nature: carbohydrates in the embryo, and those obtained from the mycorrhizal fungi (Rasmussen 1995). Some orchid seeds contain glucoproteins that may release glucose upon hydrolyzation, which could explain why some orchid seeds germinate in water (Rasmussen 1995).

Ernst and Arditti (1990) reported that *Phalaenopsis* seedlings germinated in the presence of many carbohydrate sources including glucose, a simple sugar, and maltoheptaose, a long chain sugar. Germination percentage and seedling development was highest on glucose, with fewer seeds germinating on maltooligosaccharides. Although seeds did not develop past the protocorm stage without sugar or at least a low concentration, endogenous carbohydrates must have been present to support early germination and development. After 6 months culture, seedlings cultured on glucose had higher fresh weights and survival than seedlings cultured on long-chain carbohydrates. The lower fresh weight of *Phalaenopsis* seedlings cultured with long-chain carbohydrates may be caused by insufficient enzymes responsible for breaking bonds in these carbohydrates (Ernst and Arditti 1990).

5. PHOTOPERIOD

There are several widely accepted concepts regarding the relationship between orchid seed germination and photoperiod. For example, the notion that epiphytic orchids require light and terrestrial species require darkness for germination is widely accepted. However, germination responses to photoperiods are often species specific, regardless of growth habit. Although initial germination may be greater in a particular photoperiod, protocorm and seedling development may advance more quickly under a different environmental regime. When determining an appropriate photoperiod, the growing conditions that a species encounters *in situ* should be considered with emphasis on the timing of seed dispersal. However, researchers often do not consider natural conditions when using photoperiods in germination studies. Recently, the effect of photoperiod on germination and development has been examined.

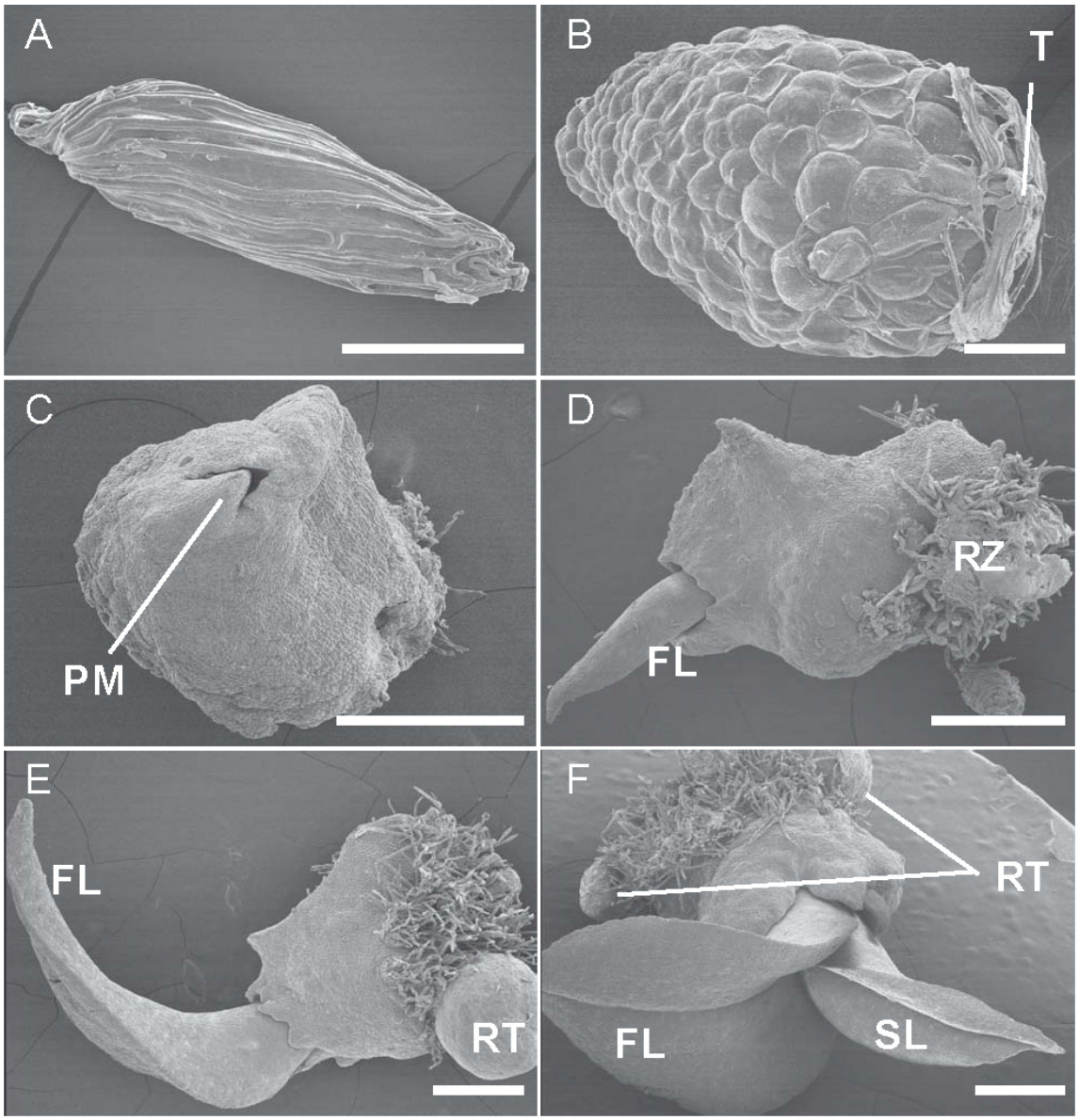

Fig. 1 Scanning electron microscopy of seed germination and subsequent development in a *Vanda* hybrid. (**A**) Stage 0 ungerminated seed; Scale bar = 100 µm. (**B**) Stage 2 protocorm; Scale bar = 100 µm. (**C**) Stage 3 protocorm; PM = protomersitem; Scale bar = 1 mm. (**D**) Stage 4 protocorm; FL = first leaf; RZ = rhizoids; Scale bar = 1 mm. (**E**) Stage 5 protocorm; RT = root; Scale bar = 1 mm. (**F**) Stage 6 seedling; SL = second leaf; Scale bar = 1 mm. (Photographic plate by T. Johnson).

Complete darkness is often considered to promote germination of terrestrial orchids. Several explanations have been offered regarding this relationship, but further studies are needed. Upon dehiscence, seeds of terrestrial orchids may not germinate until they are buried (Rasmussen and Rasmussen 1991). Many terrestrial orchids also grow in more shaded environments than their epiphytic counterparts (Rasmussen 1995), and light may not reach the habitat floor as readily (Rasmussen and Rasmussen 1991).

van Waes and Debergh (1986b) reported that even small increases in light intensity from complete darkness to 1.2 µmol m^{-2} s^{-1} reduced germination of several European terrestrial orchids. Asymbiotic germination of *Cypripedium acaule*, a North American terrestrial orchid, was lower when seeds were incubated in a 16/8 h photoperiod (6.7% germination) compared to complete darkness (96.7%) (St-Arnaud *et al*. 1992). In addition, all embryos developed leaves in darkness, but only 60% of the embryos in the 16/8 h photoperiod developed leaves (St-Arnaud *et al*. 1992). Zettler and Hofer (1997) reported a significant decrease in germination when *S. odorata* seeds were exposed to a brief period of illumination. Germination in complete darkness for three weeks was greater than germination of seeds exposed to either 7 days of an 8/16 h or 14/10 h photoperiod, and then placed in darkness for 2 weeks. Stewart and Kane (2006a) reported that light inhibited asymbiotic germination and development of *Habenaria macroceratitis*. Although protocorms developed to a leaf-bearing stage in all photoperiod treatments, over 90% of the protocorms developed leaves in complete darkness.

The aforementioned terrestrial orchids all inhabit shaded areas. *Cypripedium acaule* typically grows under shaded forests, *S. odorata* inhabits dark floodplains, and *H. macroceratitis* is found in shaded hardwood hammocks (P. Kauth, pers. obs.). In all three species, seed dispersal occurs in fall or winter when the natural photoperiod and light intensity is shorter and lower than late spring or summer. If seeds germinate upon dispersal, the reduced photoperiod as well as the shaded environment may contribute to increased germination percentages. Although seeds of each species will germinate when illuminated, a short duration of illumination is sufficient to decrease germination (St-Arnaud *et al*. 1992; Zettler and Hofer 1997; Stewart and Kane 2006a).

Protocorms cultured in complete darkness often produce more rhizoids than those in light (Stewart and Kane 2006a). Rhizoids, which are sites of fungal infections, may not be produced until seeds/protocorms are buried and likely to encounter fungal mycobionts (Rasmussen 1995). Rhizoid inhibition under light conditions may prevent protocorm death by preventing the mobilization of valuable energy reserves prior to encountering conditions of likely mycorrhizal infection (Stewart and Kane 2006a).

Stoutamire (1974) suggested that bog-inhabiting North American terrestrial orchids that are adapted to an open canopy are less sensitive to light. Kauth *et al*. (2006) found evidence for this with seeds of *Calopogon tuberosus* var. *tuberosus*, a North American terrestrial orchid. *Calopogon tuberosus* not only inhabits bogs, but also grows in areas of full sun such as open prairies and pine flatwoods. Although asymbiotic germination in complete darkness was generally greater than germination in a 16/8 h photoperiod (**Fig. 2**), seedling development was enhanced in a 16/8 h photoperiod (**Fig. 2**). No protocorms developed to an advance leaf-bearing stage under complete darkness, but over 20% of the protocorms on P723 culture medium developed to advanced leaf-bearing stages in the 16/8 h photoperiod (Kauth 2005) (**Fig. 3**). Similar results were obtained with asymbiotic germination of *Bletia purpurea*, a terrestrial orchid that grows in prairies and under open canopies in south Florida (Dutra *et al*. unpublished data). Germination and subsequent development under long day conditions may be an adaptation to shallow seed burial or germination above the substrate.

Several researchers reported that germination of orchids increases with brief periods of illumination. Rasmussen *et al*. (1990a) reported

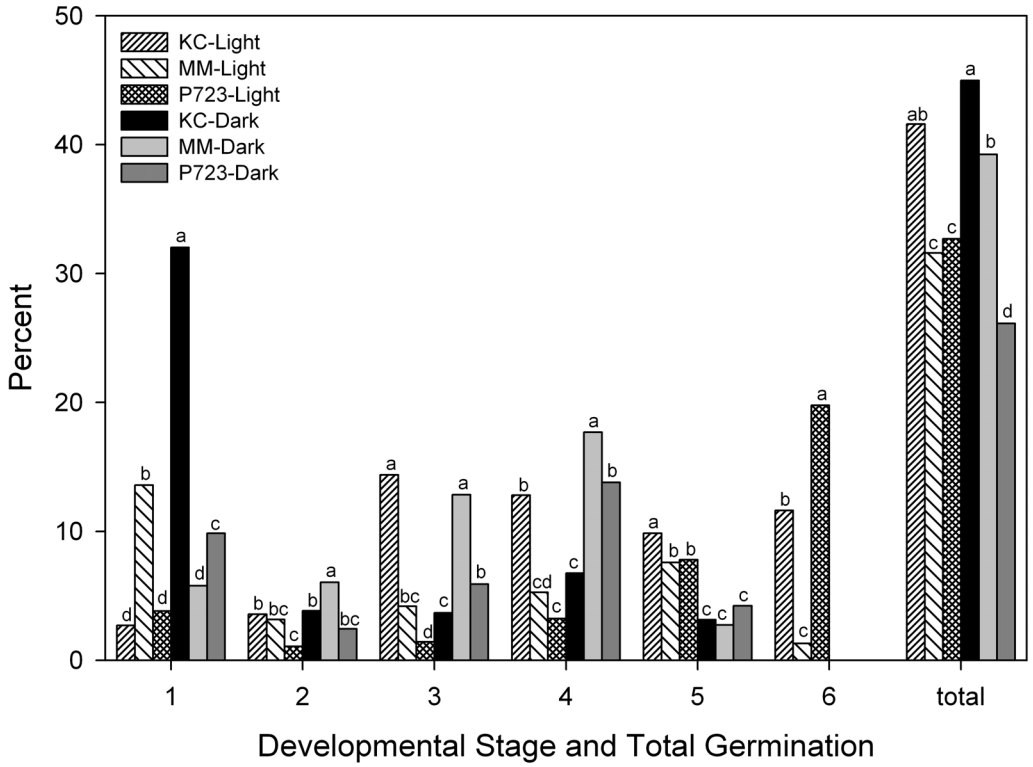

Fig. 2 Asymbiotic germination of *Calopogon tuberosus* var. *tuberosus* seeds after 8 weeks culture. Seeds were cultured in either continual darkness (Dark) or a 16/8 h L/D photoperiod (Light) for 8 weeks. Histobars with the same letter within developmental stages and total germination are not significantly different (α = 0.05). KC-Knudson C Medium; MM-Malmgren Modified Terrestrial Orchid Medium; P723-*Phyto*Technolgy Orchid Seed Sowing Medium. (Data from Kauth 2005; Kauth *et al*. 2006; with kind permission of Springer).

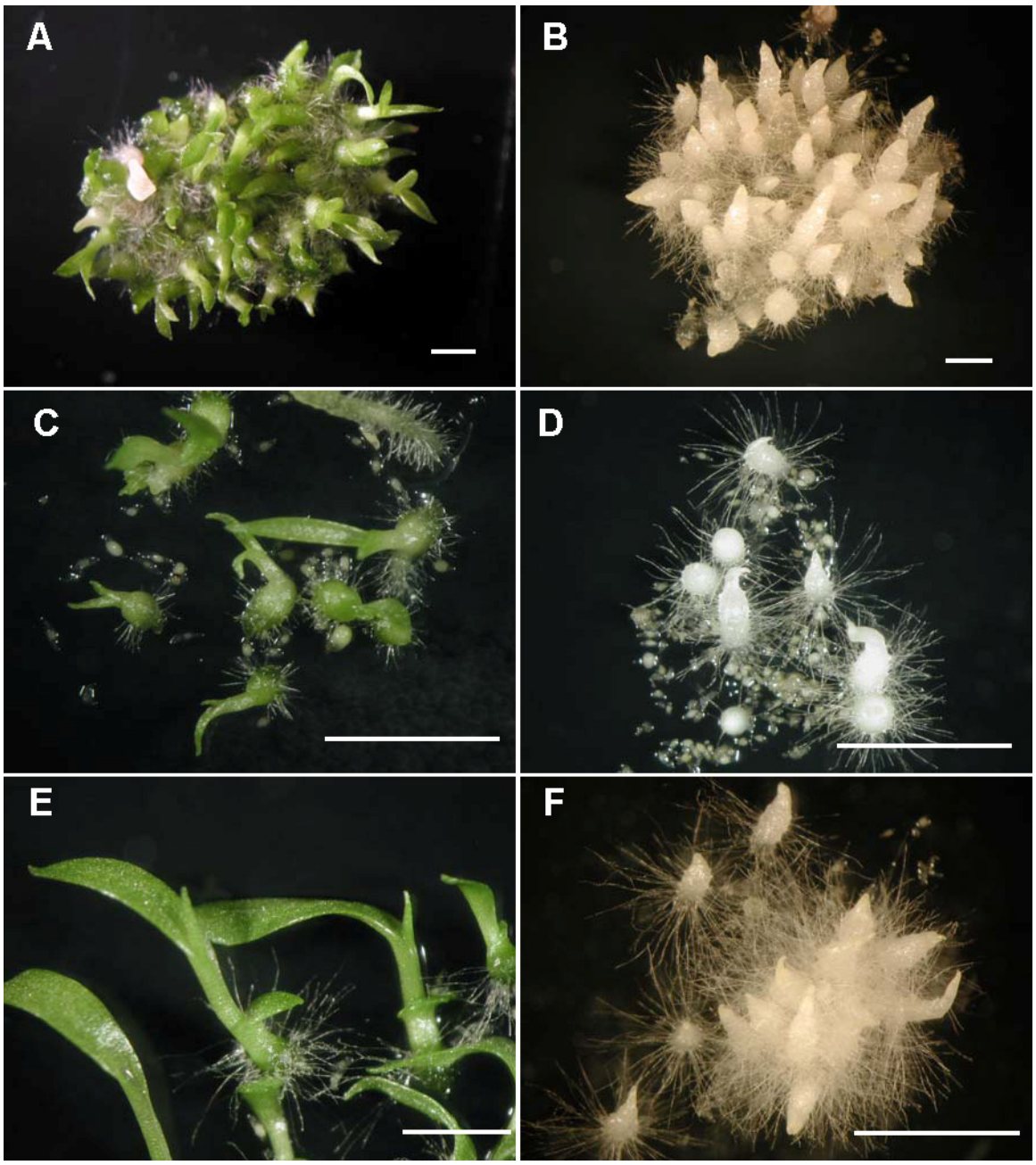

Fig. 3 Comparative effects of media and light on protocorm and seedling development of *Calopogon tuberosus* var. *tuberosus* after 8 weeks culture. (**A**) Seeds cultured on KC; 8-wk 16-h photoperiod. (**B**) Seeds cultured on KC; 8-wks continual darkness. (**C**) Seeds cultured on MM; 8-wk 16-h photoperiod. (**D**) Seeds cultured on MM; 8-wks continual darkness. (**E**) Seeds cultured on P723; 8-wk 16-h photoperiod. (**F**) Seeds cultured on P723; 8-wks continual darkness. KC-Knudson C Medium; MM-Malmgren Modified Terrestrial Orchid Medium; P723-*Phyto*Technolgy Orchid Seed Sowing Medium. Scale bars = 5 mm. (From Kauth *et al.* 2006; with kind permission of Springer).

75% germination of *Dactylorhiza majalis* when seeds were illuminated after imbibition for 10 days prior to dark incubation. This was a significant increase from 45% germination under continual darkness. Zettler and McInnis (1994) reported similar results with symbiotic germination of *Platanthera integrilabia*. Germination increased from 20% under complete darkness to 44% when seeds were exposed to 7 days of a 16/8 h photoperiod prior to dark incubation. While the exact function of light pretreatment is not understood, mycorrhizal fungi may benefit from brief periods of illumination (Zettler and McInnis 1994).

While photoperiod has been studied extensively in orchid seed germination, light quality and quantity has been generally neglected. Fukai *et al.* (1997) examined the role of light quality on asymbiotic seed germination of the hybrid *Calanthe* Satsuma. After 4 months germination percentage was highest in complete darkness (57.7%) compared to 40.2% and 1.3% germination under red and blue light, respectively. Germination was also low (12.4%) under a combination of red and blue light as well as fluorescent lights (13.2%). Blue light, although inhibitory to germination, promoted a high level of protocorm development (Fukai *et al.* 1997). Blue light has been shown to be important in photomorphogenesis as well as chlorophyll accumulation in non-orchid species (Kamiya *et al*. 1981). Likewise, red light proved beneficial for asymbiotic seed germination of *Goodyera pubescens*, and blue light and far red inhibitory (McKinley and Camper 1997). Approximately 33% germination was seen under red light and fluorescent light, while germination under blue light, UV light, and complete darkness was about 20%.

Rasmussen and Rasmussen (1991) studied the effects of light quality and quantity on symbiotic germination of *D. majalis*. Under a low white light intensity of 13 W m^{-2} (ca. 60 µmol m^{-2} s^{-1}), germination decreased from 20% in complete darkness to less than 5% (8/16 h

photoperiod) and 0% (16/8 h photoperiod). Green or red light illumination before white light decreased germination to less than 10%. However, red light followed by dark incubation increased germination to 17%.

Red light, which is physiologically active, promotes germination; however, canopies absorb red light. Red light stimulation may be an adaptation for *D. majalis* growing in open areas (Rasmussen and Rasmussen 1991). The role of phytochrome and red/far-red light has not been fully investigated in orchid seeds. Researchers experimenting with non-orchid seeds reported that red light promoted germination while seeds under far-red light did not germinate (Leon and Owen 2003; Kettenring *et al*. 2006). Experiments with non-orchid seeds may be useful as models for future orchid seed research regarding phytochrome and light quality. Although only a few published articles exist that examine light quality on orchid seed germination, more research is required on more species in order to find a definitive function of phytochrome and light quality.

6. TEMPERATURE

In practice, researchers have largely ignored the importance of temperature during orchid seed germination and seedling development. Temperatures are often selected with no justification or reference to temperatures in a species' natural range. Photoperiod is often considered more important than temperature in orchid seed germination, although research into non-orchid seed germination indicates that temperature can be more important in controlling germination (Leon and Owen 2003; Walck and Hidayati 2005). For many plant species, temperature is a major factor responsible for the onset and breaking of physiological seed dormancy (Baskin and Baskin 2004a). Baskin *et al*. (2006) recommended alternating temperature regimes for studying germination ecology of all seeds as constant temperatures are not common in nature. However, orchid seeds are often germinated *in* vitro at constant temperatures. The lack of understanding on orchid seed germination and temperature may simply be due to many studies focusing on refining methods of processing seeds, as well as understanding the nutrient requirements of symbiotic and asymbiotic germination.

Several valuable studies regarding orchid seed germination and temperature do exist. As with many other species, orchid seeds germinate within a range of temperatures, but maximum germination is achieved only in a narrow range. *Dactylorhiza majalis* seeds germinate between 10 and 30°C, but the optimum temperature range appears to be between 23 and 24.5°C (Rasmussen *et al*. 1990b). Germination percentages decreased below 15°C and above 27°C (Rasmussen *et al*. 1990b; Rasmussen and Rasmussen 1991). The development of *D. majalis* seedlings was optimum at 2-3°C below the optimal germination temperatures, and rhizoid formation was impeded above 29°C (Rasmussen *et al*. 1990b). Lower germination under symbiotic conditions at superoptimal temperatures might be due to the lack of mycorrhizal infection. Since rhizoids are the primary site of mycorrhizal infection, the lack of rhizoids may cause reduced mycorrhizal infections (Rasmussen *et al*. 1990b). Since seasonal temperatures fluctuate yearly, tolerance to a wide range of temperatures may guarantee that seeds will germinate over a period of time and not all at the same time (Rasmussen 1995).

The small size of orchid seeds and their apparent inability to germinate without exogenous nutrients makes them difficult to handle without specialized techniques. This, in turn, makes it difficult to adopt standard physiological and ecological germination techniques. However, in the case of many native Florida orchids, current studies are underway that consider the importance of seasonality, photoperiod, and temperature regime on orchid seed physiology (P. Kauth, unpublished data; S. Stewart, unpublished data). These studies will provide valuable information and contribute to the overall knowledge regarding the role of temperature in regulating orchid seed germination.

7. PLANT GROWTH REGULATORS

The use of PGRs in asymbiotic orchid seed germination has not been clarified. Cytokinins, such as benzylaminopurine (BA), zeatin (Z), thidiazuron (TDZ), and kinetin (K), often promote orchid seed germination. Auxins, such as α-naphthalene acetic acid (NAA), and ethephon, an ethylene precursor, are also commonly used to promote asymbiotic germination. Gibberellic acids (GA) also promote seed germination in many species, but the use of GA in orchid seed germination has not been very successful (Arditti 1967).

Exogenous cytokinin treatments increase asymbiotic germination of many orchid species. de Pauw *et al.* (1995), Miyoshi and Mii (1995, 1998), and Stewart and Kane (2006a) all reported increased levels of germination of several terrestrial orchids. de Pauw *et al.* (1995) reported increased germination of *Cypripedium candidum* in the presence of low concentrations of BA and 2-iP, while higher concentrations did not increase germination. A low concentration (1 µM) of naturally occurring cytokinins (zeatin and kinetin) supported a higher germination percentage of *Habenaria macroceratitis* than higher concentrations (3 and 10 µM) of zeatin, kinetin, 2-iP, and BA (Stewart and Kane 2006a; **Fig 4**). Miyoshi and Mii (1998) also reported increased levels of germination for *Cypripedium macranthos* in the presence of 1 µM kinetin.

The exact role of cytokinins in orchid seed germination is not well understood. Cytokinins in general promote cell division, as well as RNA and protein synthesis (Bewley and Black 1994). Certain mycorrhizal fungi are known to produce cytokinins (Crafts and Miller 1974). Exogenous cytokinins supplied *in vitro* may substitute for naturally occurring compounds released during mycorrhizal infection. Only two species of fungi screened produced cytokinins in appreciable amounts for detection (Crafts and Miller 1974). Other mycorrhizal fungi may produce cytokinins in amounts not detected by tests used by Crafts and Miller (1974). These low levels of cytokinins may actually better support orchid seed germination. Low cytokinin levels promoted germination further than high cytokinin levels as reported by de Pauw *et al.* (1995), Miyoshi and Mii (1995, 1998), and Stewart and Kane (2006a). Whether all mycorrhizal fungi produce cytokinins, or what type and concentration of cytokinins are optimal for asymbiotic seed germination is still not certain.

Cytokinins may also aid in lipid mobilization within orchid embryos (de Pauw *et al.* 1995). Dimalla and van Staden (1977) found that storage lipids in pecan nuts (seeds with high levels of lipids) were mobilized when treated with exogenous cytokinins. A similar process may promote orchid seed germination by utilizing lipids more efficiently in the presence of exogenous cytokinins. Using research with non-orchid seeds may help to elucidate the function cytokinins have in orchid seed germination.

Auxins stimulate ethylene evolution especially under stress conditions, which in turn stimulates seed germination in many plant species (Liberman 1979; Taiz and Zeiger 1998). Although not investigated in orchid seeds, auxins may lead to low levels of ethylene evolution. Miyoshi and Mii (1995) did not find significant increases in germination of *Calanthe discolor* in the presence of ethephon, but protocorm development advanced quickly in the presence of high levels of ethephon and auxins. Ernst *et al*. (1992) also reported no difference in germination of *Cattleya*

aurantica in the presence of ethephon, but low levels of ethephon promoted seedling development. Ethylene, released under stress conditions, might be responsible for breaking dormancy in some seeds such as sunflower and peanut (Kucera *et al.* 2005).

Miyoshi and Mii (1995) reported similar germination percentages of *Calanthe discolor* with various concentrations of BA, NAA, and ethephon, but protocorm development was more advanced when cultured in the presence of high concentrations of NAA and ethephon. Pedroza-Manrique *et al*. (2005) reported decreased germination of *Comparettia falcata* in the presence of auxins. Auxins, in the form of IAA, are not available until after germination in many seeds, and then are transported to the coleoptile tip of the seedling (Bewley and Black 1994). In monocots, such as grasses, IAA is found within the endosperm (Bewley and Black 1994), but since orchid seeds do not contain endosperm the location and function of auxins is still uncertain in orchid seed germination. However, auxins do appear to positively influence orchid seedling growth, and comparable studies with non-orchid seeds and auxins may serve as models for orchid seed research.

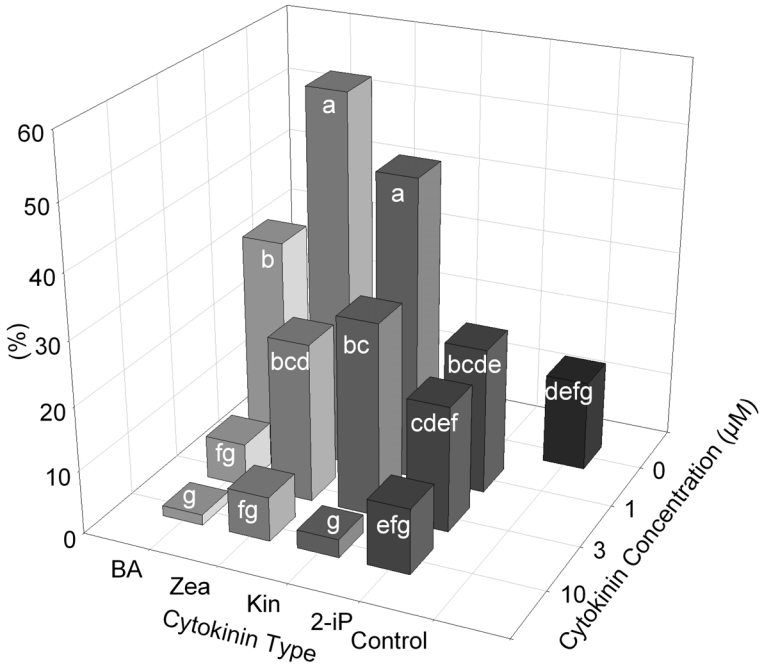

Fig. 4 Effect of cytokinin type (BA, Zea, Kin, 2-iP) and concentration (0, 1, 3, 10 µM) on percent seed germination of *Habenaria macroceratitis* after 14 weeks culture on Malmgren Modified Terrestrial Orchid Medium. BA, benzyladenine; Zea, zeatin; Kin, kinetin; 2-iP, 6-(γ,γ-dimethylallylamino) purine. (From Stewart and Kane 2006a; with kind permission of Springer).

Although GA often promotes seed germination in many plants, mixed results have been found with orchids. For example, GA_3 did not promote seed germination in *Calanthe discolor* (Miyoshi and Mii 1995), but did promote seedling development of *Phalaenopsis* (Cardenas and Wang 1998). When *Phalaenopsis* seedlings were cultured in the presence GA_3, fresh weight (613 mg) and root length (21.7 mm) were significantly greater than the fresh weight (300 mg) and root length (14.7) of the control seedlings (Cardenas and Wang 1998). The concentrations of GA_3 may have been too high to promote seed germination, but optimal for enhancing seedling development.

The role of PGRs in asymbiotic orchid germination is uncertain, and responses to growth regulators are often species specific. A major obstacle to understanding the role exogenous and endogenous PGRs have in promoting/inhibiting germination of orchid seeds may be the small size of the seeds and the possible low levels of PGRs in the embryo. Investigating the concentrations of endogenous PGRs in orchid seeds, as well as when PGRs are active in germination would greatly enhance the current knowledge of how PGRs affect germination of orchid seeds.

8. DORMANCY

Many cold-hardy terrestrial orchids exhibit low seed germination, which is often attributed to low viability or dormancy. Dormancy type and dormancy breaking mechanisms have not been studied in depth in the Orchidaceae. Morphological, physiological, and morphophysiological dormancy have all been identified in orchid seeds (Baskin and Baskin 2001). Morphological dormancy is characterized by a delay in germination due to an undifferentiated embryo in orchid seeds (Baskin and Baskin 2001). However, morphological dormancy is difficult to characterize in orchids since testa rupture and germination may occur when the embryo is still relatively undifferentiated. Since extensive embryo development prior to testa rupture may not be necessary, the tenant of morphological dormancy as it is currently accepted may not directly relate to orchids. In physiological dormancy, the embryo does not have sufficient growth potential to break through the testa, but imbibition occurs (Baskin and Baskin 2001). Recent studies reported that physical dormancy may delay germination of various taxa in the Orchidaceae, especially cold-hardy terrestrials (Lee *et al*. 2005, 2006; Yamazaki and Miyoshi 2006). Physical dormancy is similar to physiological dormancy, but physically dormant seeds are unable to imbibe water due to the hydrophobic nature of the testa (Baskin and Baskin 2004b). Although dormancies do contribute to low germination percentages in orchid seeds, seed age and seed viability should be investigated before low germination is attributed to dormancy.

8.1. Seed viability

Attempts have been made to link low germination and low viability through tetrazolium (TZ) testing. In a TZ test, viable and respiring embryos are stained red and nonviable embryos remain uncolored (Lakon 1949). Mixed results were obtained with TZ tests of Western European Orchids (van Waes and Debergh 1986a). Of 16 species examined, only four were successfully stained using classic TZ procedures. These variable results were attributed to the inconsistent permeability of the different species' testas. Impermeable testas are common in terrestrial orchids, while epiphytic orchids often have dry cracks in the testa (van Waes and Debergh 1986a). The testas of several *Vanda* hybrids degrade after two minutes in sodium hypochlorite (T. Johnson, pers. obs.), while seed of several *Cypripedium* species can withstand several hours in a hypochlorite solution (Steele 1996). Suberin, a waxy substance, is commonly found on testas of orchid seeds, which may contribute to impermeability of the testas. After testing for viability and determining seeds are viable, dormancy may be a contributing factor to low seed germinability.

8.2. Seed age

Immature seeds of many orchid species have been shown to germinate more readily than mature seeds (Arditti *et al*. 1981; Arditti 1982; Linden 1992; de Pauw and Remphrey 1993). Several factors may contribute to the increased germinability of immature seeds. Immature seeds may be more water permeable than mature seeds (van Waes and Debergh 1986b). Mature seeds may have chemical inhibitors, such as ABA, or lack certain germination promoting hormones (van Staden *et al*. 1972; van der Kinderen 1987), or have an impermeable testa (Yamazaki and Miyoshi 2006).

The low germinability in mature seeds of several species has been linked to the development of the testa. St-Arnaud *et al*. (1992) reported higher germination in seeds of *C. acaule* collected 60 days after pollination (DAP) than seeds collected 30 or 90 DAP. de Pauw and Remphrey (1993) found that germination of three *Cypripedium* species improved when seeds were collected 8 weeks after pollination (WAP), and declined rapidly thereafter. Germination of *Dendrobium tosaense* seeds improved 8 and 9 WAP compared to 10-14 WAP (Lo *et al*. 2004). Yamazaki and Miyoshi (2006) reported higher germination with seeds collected 70 DAP in *Cephalanthera falcata*, and decreased germination thereafter. The decreased germination with seeds collected 80 DAP, and thereafter, was attributed to lignification and cutinization of the integument that created an impermeable layer. Seeds harvested at least 140 DAP did not stain in a TZ test unless the integument was degraded through chemical scarification. Yamazaki and Miyoshi (2006) found that the TZ solution did not penetrate the integument 100 DAP, and thereafter. This indicates that after 100 DAP the integument provides a significant barrier to water uptake, and contributed to delayed germination. Rasmussen and Whigham (1993) found that seeds of several terrestrial orchids can remain in the soil for months or years. The impermeable nature of the testa may contribute to a persistent seed bank. This is an advantage to species whose seeds are dispersed at times for unfavorable germination, and are able to survive until conditions are met for germination (Rasmussen 1995).

Yeung *et al*. (1996), Lee *et al*. (2005), and Lee *et al*. (2006) found that cuticular substances around the inner integument and embryo caused a hydrophobic barrier to water and nutrient uptake in *Cymbidium sinense*, *Cypripedium formosanum*, and *Paphiopedilum delenatii* seeds, respectively. Lee *et al*. (2005) found that 135 DAP the inner integument shrunk and formed a tight layer around the embryo, resulting in poor germination. At 150 DAP the cuticular substances started to form a complete layer around the embryo proper, and at full maturity (210 DAP) the cuticular substances enveloped the entire embryo (Lee *et al*. 2006). At 105 DAP the outer layer of the testa began to shrink and compress the embryo, and at full maturity the testa was completely dehydrated and formed a tight barrier around the embryo (Lee *et al*. 2006). Although cuticular substances were located in the testa, the suspensor region was free of these substances, allowing nutrients and water to move into the embryo (Lee *et al*. 2006). The impermeable nature of the testa indicates the presence of physical dormancy. Since the suspensor region may be a channel for imbibition, the role of physical dormancy, which appears to develop at a specific time, is still not well-understood. To fully examine the role of physical dormancy, imbibition rates of individual seeds must be examined. However, weighing individual orchid seeds is a difficult task given the equipment required to accomplish this, such as a highly sensitive electrobalance.

8.3. Cold-stratification

Several treatments are effective at breaking dormancy in mature orchid seeds including cold-stratification, ultrasonic treatments, and chemical treatments. The use of cold-stratification for dormancy breaking in orchid seeds is often used for difficult-to-germinate genera such as *Cypripedium*, *Epipactis*, and *Dactylorhiza* (Rasmussen 1995). However, there is limited information on the exact mechanism by which cold-stratification promotes orchid seed germination. Cold temperatures may decrease enzymatic reactions, slow metabolic processes, or change enzyme production and concentration (Bewley and Black 1994). Metabolic processes that inhibit germination may be slowed during stratification allowing germination to proceed (Bewley and Black 1994).

The majority of cold-stratification research on orchid seeds has been conducted on *Cypripedium* species. However, variable results have been reported not only between species, but also within the same species. Ballard (1990) reported a maximum germination in *Cypripedium calceolus* of 16% after 4 months of cold-stratification at 5°C, while Coke (1990) reported 50% germination after 5 months of cold-stratification. In a parallel study, germination of *C. calceolus* increased to over 90% after cold-stratification at 5°C for 8 weeks (Chu and Mudge 1994). In a different study pretreatment of *C. calceolus* seeds at 6°C for 8 weeks reduced germination to 1.6% (van Waes and Debergh 1986b). Different capsule ripening conditions and seed age may have caused the different results. van Waes and Debergh (1986b) used fully mature seeds collected from dehisced capsules, while Chu and Mudge (1994) used non-dehisced mature seeds. Dehisced seeds may need a longer period of cold-stratification than van Waes and Debergh (1986b) provided. Since Chu and Mudge (1994) cultured seeds in complete darkness while van Waes and Debergh cultured seeds under a 14/10 h photoperiod (1986b), differences in germination may be attributed to other culture conditions.

The length of cold-stratification is also an important factor to consider, and may be species specific. Rasmussen (1992), Tomita and Tomita (1997), and Miyoshi and Mii (1998) reported higher germination percentages when seeds of *Cypripedium macranthos*, *C. candidum*, and *Epipactis palustris*, respectively, were cold-stratified for 8 to 12 weeks. Zettler *et al*. (2001) found that germination percentage of *Platanthera leucophaea* increased after two cold-stratifications for 11 months as well as 107 days at 6°C following 95 days at 23°C. Sharma *et al*. (2003) reported a higher germination percentage of *Platanthera praeclara* after 6 months of cold-stratification compared to 0 and 4 months. Shimura and Koda (2005) reported the importance of fungal inoculation corresponding to cold-stratification on symbiotic germination of *C. macranthos*. A higher germination percentage was reported when seed cultures were inoculated with fungi after a 12 week cold-stratification compared to inoculation before or several weeks after the cold-stratification. This might suggest that fungal infection in nature takes place after winter and prior to germination in early spring (Shimura and Koda 2005).

Cytokinins may also contribute to breaking dormancy in many seed types, and research in this area may serve to elucidate their role in orchid seed germination. During cold stratification, endogenous cytokinin levels in the embryo for many non-orchid species increase (Bewley and Black 1994), thus possibly substituting for cold stratification (Miyoshi and Mii 1998). In *Acer saccharum* seeds, kinetin levels increased significantly after incubation for 20 days at 5°C, but kinetin was not found in seeds incubated at 20°C (van Staden *et al*. 1972). Cytokinins also increase during seed development and seed tissue growth but decline with seed maturation (Bewley and Black 1994). Little to no kinetin was found in *Acer saccharum* seeds that were incubated for 20 days or already germinated (van Staden *et al*. 1972). The role of cytokinins as well as

cytokinin types in orchid seed germination should be tested using both mature and immature seed.

Cold-stratification in orchid seeds has several ecological functions and effects. If seed dispersal occurs in fall, seed germination may be delayed until the next growing season when conditions are more favorable for growth and development. A low temperature requirement may prevent seeds from germinating immediately after dispersal (Rasmussen 1995). *Calopogon tuberosus* seeds from northern populations germinate quickly and allocate more biomass to storage organs. This mechanism may allow seeds and seedlings to survive winter months successfully (P. Kauth, unpublished data). The effects of chilling and thawing may cause degradation of the testa, which could lead to leaching of germination inhibitors, imbibition, and fungal infection (Rasmussen 1995). Chilling also promotes the growth of rhizoids, which are important for the uptake of water and nutrients as well as establishing the mycorrhizal fungal relationship (Rasmussen 1992).

8.4. Seed coat treatments and hormonal inhibitors

Treatments, such as hypochlorite soaks, can be used to weaken the testa, improve permeability, and promote germination. These treatments are often used to bypass physiological or physical dormancy. Miyoshi and Mii (1998) reported increased germination after pretreating seeds of *Cypripedium macranthos* with 0.5% sodium hypochlorite or 3.2% calcium hypochlorite. However, higher concentrations of hypochlorite and long periods of presoaking may lead to decreased germination by damaging embryos. Care must be taken to find the optimal surface disinfecting solution and appropriate time to optimize germination.

One technique not widely utilized for degrading the testa is sonication. *Calanthe discolor* seed germination increased under sonication (Miyoshi and Mii 1988). Sonication removed testas after 4 minutes, and all embryos were free of the testa after 12 minutes. However, embryos were damaged by 8 minutes of sonication. Lauzer *et al*. (1994) also used sonication to improve germination of *Cypripedium acaule*. At 3.5 minutes approximately 30% of the embryos lost their testas compared to about 70% of the embryos in the 7 minute treatment. However, the 7 minute treatment decreased germination when compared to the 3.5 minute treatment. As reported by Miyoshi and Mii (1988), prolonged sonication damaged the embryos, perhaps causing decreased germination.

Abscisic acid (ABA) has been studied in many species; however, the role of ABA in orchid seed germination has not been extensively studied. ABA is known to prevent germination and induce seed dormancy in many plant species (Bewley and Black 1994). ABA is synthesized both in the embryos and testas of many non-orchid species, and serves to prevent germination when seeds do not have sufficient nutrient reserves (Kermode 2005). It also accumulates as seeds mature, and levels peak at mid-maturation of seeds (Kermode 2005). ABA was found in two species of terrestrial European orchids, but was not assumed to be the sole contributor to dormancy (Van der Kinderen 1987). Whether ABA affects seed germination depends on the location and the sensitivity of the cells to ABA (van Der Kinderen 1987). Free ABA levels were higher in mature seeds (7.2 µg/g fresh weight) of *Dactylorhiza maculata* than in immature seeds (0.514 2 µg/g fresh weight). This study showed that ABA accumulates in maturing embryos, but the exact point of peak accumulation was not stated.

In a very important study, Lee *et al*. (2007) studied the role of ABA in seed germination of *Calanthe tricarinata*. ABA levels remained low from 60-90 DAP at 2.16-2.26 $ng \cdot mg^{-1}$ fresh weight. However, as seed age increased the ABA levels also increased. At full seed maturity of 210 DAP, ABA levels peaked at 11.6 $ng \cdot mg^{-1}$ fresh weight. They also reported that pretreatment of mature seeds with ultrasound, 1% NaOCl, or 1 N NaOH for 15-60 minutes improved germination and decreased ABA levels. After 15 minutes of pretreatment, ABA levels were as follows: 11.1, 8.7, and 7.4 $ng \cdot mg^{-1}$ of fresh weight for ultrasound, 1% NaOCl, and NaOH, respectively. After 60 minutes of pretreatment ABA levels decreased to 6.2, 2.6, and 1.8 $ng \cdot mg^{-1}$ of fresh weight for ultrasound, 1% NaOCl, and NaOH, respectively.

Seed pretreatments may improve orchid seed germination by changing the physical characteristics of the testa (Lee *et al*. 2005). Sonication and chemical treatments cause ruptures in the testa resulting in increased water and nutrient uptake by the embryo (Miyoshi and Mii 1988). Removing the embryo from the testa removes possible germination inhibitors in the testa; however, identification of possible chemical inhibitors in the testa has not been fully investigated. Chilling or soaking might lead to leaching of chemical inhibitors such as ABA that develop during seed maturation (Linden 1992). In non-orchid species, chilling has been shown to decrease ABA (Feurtado *et al*. 2004), but more research is required with orchids.

9. SYMBIOTIC GERMINATION

Although many common factors, such as photoperiod and temperature, influence both asymbiotic and symbiotic germination, the two methods are quite different. Symbiotic germination techniques were developed prior to asymbiotic germination; however, asymbiotic germination methods are more widely used at present. Since fungi are utilized in symbiotic germination, additional training beyond asymbiotic germination techniques is required (Zettler 1996). For this reason, symbiotic germination is often unjustly considered more difficult or more complicated than asymbiotic germination (Zettler 1996). The popularity of asymbiotic germination has, until recently, caused a lack of orchid-fungal symbiosis research (Zettler 1997a). To date, most symbiotic germination research has emphasized temperate terrestrial orchids that are often difficult to germinate in asymbiotic culture (Zettler 1996).

9.1. Symbiotic techniques

The most important step in symbiotic germination is the isolation and identification of root inhabiting mycobionts. The basic procedure for isolating mycobionts is to harvest roots from orchid plants and isolate the fungi under *in vitro* conditions. Orchid roots are harvested, rinsed in cold tap water, and surface sterilized (Zettler 1997b). Roots can be either macerated and inoculated onto an appropriate culture medium, or individual root-inhabiting fungal structures, called pelotons, can be isolated and placed onto a culture medium (Zettler 1997b; Stewart and Kane 2006b). To promote fungal growth, fungi are inoculated onto a medium rich in nutrients [Potato Dextrose Agar (PDA; BD Company, Sparks, MA) or Corn Meal Agar (CMA; Sigma-Aldrich, St. Louis, MO); **Table 3**] that can be supplemented with antibiotics to inhibit bacterial growth (**Fig. 5**). After 2-5 days, the tips of developing fungal cultures are excised and subcultured (Stewart and Kane 2006b). After several more days, fungi are identified and stored at 10°C on oat meal agar (Dixon 1987; **Table 3**).

Although the symbiotic technique of germinating orchid seeds is easy to implement in theory, there are several drawbacks to this method.

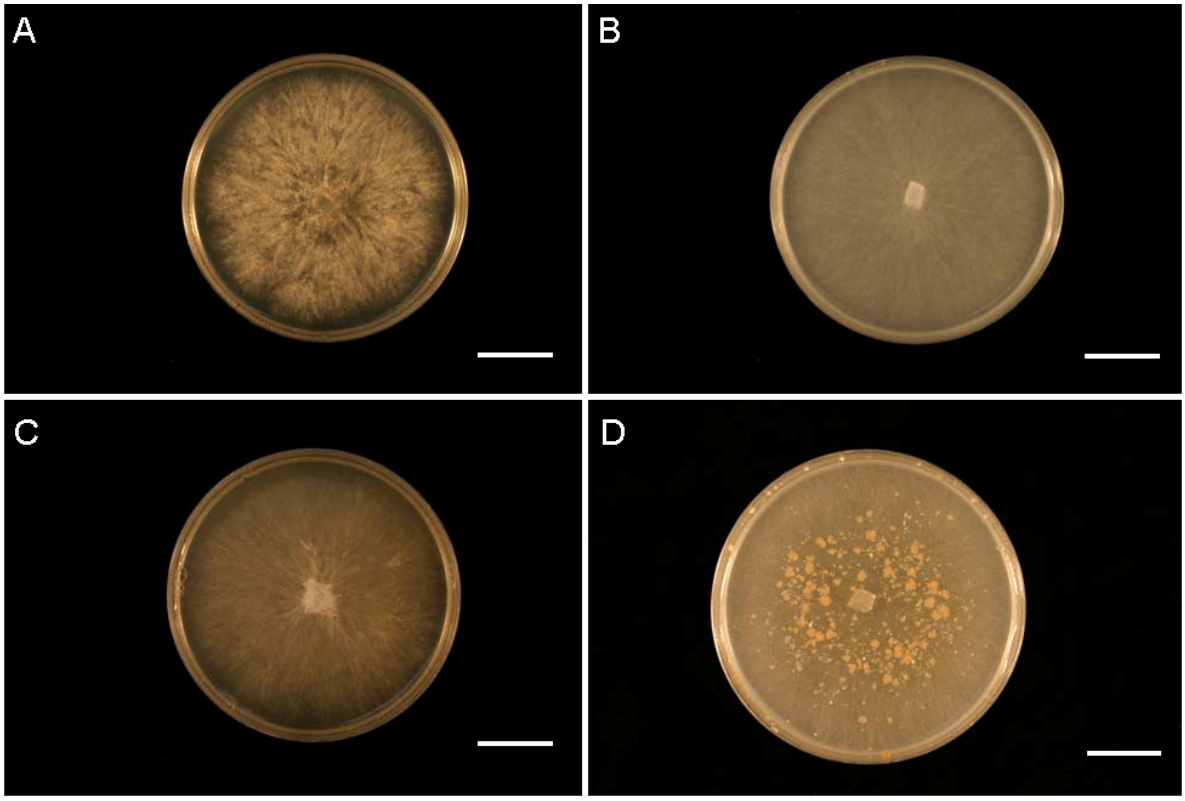

Fig. 5 Typical orchid mycobionts in pure culture growing on PDA after 20 d incubation. (**A**) Unidentified Basidiomycotina species isolated from *Eulophia alta*, a terrestrial orchid in south Florida. (**B**) *Epulorhiza repens* isolated from *Spiranthes brevilabris*, a terrestrial orchid in central Florida. (**C**) *Epulorhiza repens* isolated from *Habenaria macroceratitis*, a terrestrial orchid in central Florida. (**D**) Unidentified *Ceratorhiza* species isolated from *Spiranthes floridana*, a terrestrial orchid in northern Florida. Scale bars = 2.25 cm. (Pictures by S. Stewart).

Fungal succession may be responsible for germination and subsequent development. Younger plants and seedlings may better support fungi responsible for germination, while fungi isolated from mature plants may better support development (Zettler 2001; Rasmussen 2002; Sharma *et al.* 2003). *In vitro* seed germination of *Platanthera praeclara* increased when seeds were co-cultured with a fungus isolated from seedlings and protocorms compared to mycobionts isolated from mature plants (Sharma *et al.* 2003). In addition, fungal colonization in orchid roots may be variable throughout the year, as well as location of the fungal infection in the orchid root system (Zettler 2001). Also individual pelotons may host several fungal species, many of which may not be responsible for germination (Zettler *et al*. 2003).

Table 3 Formulations of symbiotic germination media per 1 liter water: Corn Meal Agar (CMA); Oat Meal Agar (OMA) (Dixon 1987); Potato Dextrose Agar (PDA).

Component	CMA	OMA	1/5th PDA
Pulverized oats		3.0 g	
Corn meal	50 g		
PDA powder			6.8 g
Yeast extract		100 mg	
Bacto-agar	15.0 g	7.0 g	6.0 g

In order to increase the chances of isolating mycobionts that promote germination, nylon mesh bags containing seeds are often used for *in situ* germination (Rasmussen and Whigham 1993). For this technique, nylon mesh packets (4 x 6 cm; 35 µM pore size) are inoculated with seeds. The pore size must be small enough to retain the seeds, but large enough to allow fungal hyphae penetration. This method is sometimes termed fungal baiting, since the seed packets are used to attract fungi that promote germination (Brundrett *et al*. 2003). In order to isolate mycobionts from the packets, the protocorms are removed from the packets, surface sterilized, and macerated (Zettler *et al*. 2005b). Nutrient medium is then poured over the macerated protocorms to allow mycobiont growth (Zettler *et al*. 2005b). This technique may ensure the isolation of mycobionts that promote germination.

9.2. Fungal specificity

Although symbiotic germination may result in higher germination percentages than asymbiotic germination (Rasmussen *et al*. 1990b; Zettler and McInnis 1994), controversy exists whether orchid seeds require specific fungi to fully promote germination and subsequent development (Rasmussen 2002). *In vitro* fungal specificity appears to be highly specific for some species. Zettler *et al*. (1999) reported 100% germination of *Encyclia tampensis* seeds when using a fungal isolate from *Epidendrum conopseum*, but few seedlings developed to a leaf-bearing stage. Otero *et al*. (2004) reported high germination in two epiphytic orchids when using fungal isolates from each orchid. Fungi isolated from both *Tolumnia variegata* and *Ionopsis utricularioides* supported seed germination from both species. However, seed germination and subsequent development of *I. utricularioides* increased when co-cultured with its own mycobionts, but germination was still over 60% when co-cultured with *T. variegata* isolates (Otero *et al*. 2004). *Tolumnia variegata* was found to be a generalist while *I. utricularioides* was more specific in its mycobiont preference. *Ionopsis utricularioides* has a more restricted geographic range than *T. variegata*, therefore it may have a more specific mycobiont requirement than *T. variegata* (Otero *et al*. 2004).

Terrestrial orchids appear to have a higher degree of mycobiont specificity at the generic and species levels. Although *Platanthera integrilabia* seeds did germinate with mycobionts from other *Platanthera* species, only isolates from *P. integrilabia* supported advanced seedling

development (Zettler and McInnis 1992). Zettler and Hofer (1998) found that mycobionts from four *Platanthera* species supported germination of *P. clavellata*. Since *Platanthera* species are often found growing in close proximity, these species might utilize similar mycobionts to support germination, but not subsequent development (Zettler and Hofer 1998). Similarly, isolates from both *Spiranthes brevilabris* and *S. floridana* supported germination of *S. brevilabris* seeds; however, only mycobionts from *S. brevilabris* supported subsequent development (Stewart and Kane 2007). *Spiranthes brevilabris* is a rare Florida terrestrial orchid, and its rarity may be attributed to the high mycobiont specificity of the species (Stewart and Kane 2007).

As previously mentioned, fungal succession may be more prevalent than formerly believed. This fungal succession from seed to seedling to mature plant remains unclear. However, recent findings may help to clarify this mystery. Two antifungal compounds were isolated during the symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Shimura *et al.* 2007). The first compound, lusianthrin, was isolated when protocorms were inoculated with a mycorrhizal fungus. Chrysin, the other antifungal compound, was only isolated in non-infected protocorms. A large increase in pelotons occurred simultaneously with an increased level of lusianthrin, and after 4 months lusianthrin levels were twice the ED_{100}, but hyphae and pelotons were still found in cytoplasm cells. Lusianthrin was determined to maintain the balance between the symbiotic relationship involved in seed germination. Because chrysin was not isolated in protocorms, this antifungal compound may only be produced by adult plants. Shimura *et al*. (2007) determined that orchids utilize multiple antifungal compounds at certain stages of development.

Symbiotic seedlings cultured with fungi from other species or locations may not be suitable for reintroduction, and raises ecological concerns. Introducing a non-native mycobiont into a new site may have consequences similar to introducing plants into non-native habitats (Zettler *et al*. 2003, 2005a; Stewart and Kane 2007). Non-native fungi may not have the capacity to survive or support the growth of a host plant in a foreign habitat, thus causing the reintroduced plants to die. Different strains within a fungal species may cause harm and permanent damage to isolated ecosystems or other rare and endangered plants (Zettler *et al*. 2005a). Likewise many mycorrhizal fungi are *Rhizoctonia*-like fungi, which can be parasitic under some conditions. In addition, introducing non-native fungi to a reintroduction site may have detrimental effects on the habitat by releasing a potentially pathogenic fungus or interfering with the balance of native biota.

9.3. Mycobiont contributions to orchid seeds

Although orchids do require a mycorrhizal association for *in situ* germination and subsequent development, the role of the fungus is still not well-understood. Mycobionts provide orchid embryos with water, nitrogen, carbohydrates, vitamins, and undefined organic compounds (Yoder *et al*. 2000; Rasmussen 2002). To gauge the success of symbiotic germination, direct comparisons between asymbiotic and symbiotic germination should be investigated. However, direct comparisons between the techniques are questionable since the culture media are very different (Rasmussen *et al*. 1990b). Several direct comparisons between symbiotic and asymbiotic germination of terrestrial orchids do exist. Although different species were studied, evidence suggests an advantage for symbiotic seed germination and seedling development (Rasmussen *et al*. 1990b; Rasmussen 1992; Oddie *et al*. 1994; Zettler and McInnis 1994; Takahashi *et al*. 2000; Johnson *et al.* 2007). The components of asymbiotic media, although suitable for germination, may not substitute for the mycobiont or the nutrients made available to the embryo by the mycobiont upon infection (Rasmussen 1992).

Supplying water to germinating orchid seeds may be an overlooked yet important contribution of the mycobiont. Seeds of *Platanthera integrilabia*, a terrestrial orchid, and *Epidendrum conopseum*, an epiphytic orchid, exhibited increased water content when cultured symbiotically (Yoder *et al*. 2000). Water content, as well as the ability to retain water, was greater in *P. integrilabia* seeds than *E. conopseum* seeds. Since the seeds of *E. conopseum* were smaller in size than those of *P. integrilabia*, a large surface to volume ratio existed, which was responsible for the higher water loss. This suggests that the testa of *E. conopseum* was not as water impermeable as that of *P. integrilabia*, which may be the reason epiphytic orchids germinate more readily *in vitro*. Terrestrial orchids may more commonly have a hydrophobic testa in order to avoid imbibition under unfavorable germination conditions (Rasmussen and Rasmussen 1991).

Nitrogen also is an important nutrient in the mycorrhizal-orchid relationship. A limited concentration of nitrogen and high carbohydrate supply promoted mycobiont infection and increased germination of *Orchis morio* seeds (Beyrle *et al*. 1995). A high concentration of nitrogen and low carbohydrate supply did not result in mycobiont infection, but rather led to parasitism by the fungus on the orchid seeds. High nitrogen content in the culture medium caused a cell wall thickening and accumulation of phenolic compounds. This thickening of the cell wall, in turn, may be difficult for a potential mycobiont to penetrate and establish an association with the orchid seed (Beyrle *et al*. 1995). The requirement of low nitrogen, potassium, and phosphorus for adult plants of *Dactylorhiza majalis* (Dijk and Olff, 1994) may be due to the competitive nature of mycorrhizae on low nutrient soils (Rasmussen 1992).

10. SEED SOURCE

One purpose of orchid seed germination is to provide plants for species-level conservation and reintroductions. However, populations of one species may inhabit strikingly different habitats across a geographic range. For example, *Calopogon tuberosus* inhabits alkaline, mesic prairies in south Florida and acidic bogs throughout its northern range into Canada. These habitats may alter the genotypic and/or phenotypic compositions of this orchid producing distinct ecotypes adapted to local environmental conditions (Hufford and Mazer 2003). Introducing inappropriate ecotypes into a particular habitat could not only lead to the death of transplanted individuals, loss of genetic diversity, and population degradation. With an increasing interest in orchid-species conservation, care must be taken to use local seed. Although no previous studies exist that differentiate orchid ecotypes, compare seed germination among ecotypes, or characterize seed germination and development to habitat differences, this area of research is currently ongoing (P. Kauth, unpublished data).

Several studies do exist that compare seed germination among different seed sources of the same species. Zettler and McInnis (1992) reported germination differences between seed sources of *Platanthera integrilabia*. The highest germination percentage and seedling establishment was observed in seeds from the largest known population of *P. integrilabia*, while seed collected from smaller populations was found to have lower germination percentages and seedling establishment (Zettler and McInnis 1992). Inbreeding depression in smaller populations could lead to differences in germinability (Zettler and McInnis 1992), low viability, or reduced vigor. Zettler and Hofer (1998) reported

differences in germination among populations of *Platanthera clavellata*. Although seed originating from Georgia had lower germination than other sources, seedling development was superior with Georgia seeds (Zettler and Hofer 1998). Since *P. clavellata* is an auto-pollinated species, it may be likely that small differences in seed viability or genetic diversity would occur between populations (Zettler and Hofer 1998). Although habitat conditions were not incorporated into this study, the size of the populations and apparent isolation may have caused genetic differences in seed germination. Recently the symbiotic germination between two populations of *Epidendrum nocturnum* was examined (Zettler *et al.* 2007). Across all treatments germination of seeds from Fakahatchee Strand State Preserve in Florida averaged 55.7% while germination was 12.7% on average from seeds from the Florida Panther National Wildlife Refuge (FPNWR). However, seeds from the FPNWR had a viability of 79.7% compared to a viability of 72.6% for Fakahatchee seeds. Although seed handling and age may have contributed to these differences (Zettler *et al*. 2007), the self-pollinating breeding system may have also contributed to the germination and viability differences.

Dijk and Eck (1995b) investigated the role of *in vitro* seedling mineral nutrition between coastal and inland populations of *Dactylorhiza incarnata* in the Netherlands. Major differences in seed germination responses to nitrogen type and population location were noted. Seedlings from coastal areas grew faster *in vitro* and were more tolerant of exogenous ammonium and nitrate, while the inland seedlings were more sensitive to both ammonium and nitrate. However, seedlings from both populations were more sensitive to high concentrations of exogenous nitrogen. Since the coastal seedlings developed quickly, they were also able to assimilate nitrate more efficiently. Both populations inhabit calcareous areas where high nutrient levels are found due to the introduction of fertilizers and poor drainage. These soil conditions have led to decreased *D. incarnata* plant numbers. Increased nitrogen mineralization inland may have caused increased nitrogen sensitivity of these plants. Although Dijk and Eck (1995b) were uncertain whether habitat influenced developmental differences, habitat differences seem to have influenced the ecotype differentiation as shown by the observed differences in seedling development.

Possible ecotypic differentiation among populations in other orchid species has been observed. Preliminary results showed major differences in asymbiotic germination among populations of *Calopogon tuberosus* when comparing photoperiods (P. Kauth, unpublished data). Under short days germination percentages of north central and south Florida seeds were higher than neutral and long days, while no difference in germination of Michigan seeds was seen among photoperiods. Florida seeds had the highest germination percentage compared to South Carolina and Michigan seeds. Seedling corm development also differed under *in vitro* conditions. While south Florida seeds did not form corms *in vitro* within 16 weeks, seeds from north-central Florida, South Carolina, and Michigan formed corms readily. Higher biomass allocation to corms and rapid corm formation was observed in Michigan seedlings compared to all other seed sources. The tendency to form corms quickly may be caused by a shortened growing season. Seeds from more northern populations may germinate and form corms immediately in order to survive winter conditions. As in the south Florida seedlings, corm formation in seedlings from extreme southern populations may be slower because of the longer growing season.

Additionally, germination percentages were different on various culture media (P. Kauth, unpublished data). Culture media with high concentrations of micronutrients promoted germination and seedling development in Florida and Michigan seeds. Media with high mineral salt concentrations and low micronutrients promoted seed germination of South Carolina seeds. Differences in germination on different culture media among *C. tuberosus* populations may be the result of soil nutrient availability at each site, or the result of ecotypic development caused by different photoperiod, temperature, seed viability, and genetic diversity (P. Kauth, pers. obs.).

11. ACCLIMATIZATION

Ex vitro survival of orchid seedlings is often low, and improving the survival of orchid seedlings *ex vitro* is essential for reintroduction programs (Zettler *et al*. 2005b). Terrestrial orchid seedlings often do not survive after the first growing season in the field due to a lack of storage organs such as tubers and corms, or because of storage organ mortality (Batty *et al*. 2006a; Scade *et al*. 2006). To increase seedling survival during *ex vitro* transfer, seedling acclimatization may be necessary for some species (Zettler *et al*. 2005b; Batty *et al*. 2006a). Since *in vitro* grown seedlings often have low or no stomatal activity (due to continually high humidity) and are grown in a high nutrient environment, acclimatization procedures are used to gradually decrease relative humidity levels, increase photosynthetic capacity, and acclimate seedlings to low nutrient environments (Batty *et al*. 2006a). However, research on the photosynthetic rates of *in vitro* orchid seedlings during acclimatization is rare.

Zettler *et al*. (2005b) and Batty *et al*. (2006a) utilized methods to increase seedling survival of terrestrial orchids by transferring symbiotically grown seedlings to larger culture vessels containing a layer of symbiotic culture medium, sand, and charcoal. These larger culture vessels provide seedlings and mycobionts with a fresh source of carbohydrates, a substrate to absorb possible growth-inhibitors, and a gas-permeable environment provided by small pore-sized nylon mesh disks. Decreased humidity, increased moisture loss, and increased gas exchange were the primary reasons for successful seedling survival (Zettler *et al*. 2005b; Batty *et al*. 2006a). Scade *et al*. (2006) reported successful seedling survival of several Australian terrestrial orchids as well as the reintroduction of these seedlings into natural habitats using this technique. However, long term survival depended several *ex vitro* environmental factors including weed coverage, canopy cover, and site slope (Scade *et al*. 2006).

Another technique to increase survival of field transplanted seedlings is to use dormant organ structures as propagules (Batty *et al*. 2006b). Dormant tubers of several Australian orchids survived for longer periods of time than did seedlings after transplantation, since the tubers contained more nutrient reserves, and were able to survive harsh environmental conditions better than seedlings. After five growing seasons, 80% of *Diuris micrantha* tubers survived. Other taxa had lower survival compared to *D. micrantha*; however, the use of dormant tubers for transplanting generally provided the highest survivorship after five years. Large tubers of *D. micrantha* survived dormancy and subsequently flowered after 2 years under natural conditions, while mortality increased with smaller tubers. The larger tubers may have contained greater nutrient reserves to survive dormancy and initiate growth (Batty *et al*. 2006b).

Those species that do form storage organs often do not form them readily *in vitro*. Several studies have shown that dormant structures can be induced *in vitro*. Stewart and Kane (2006a) and Tissue *et al*. (1995) reported increased tuberization and corm formation under short days with the North American orchids *Habenaria macroceratitis* and *Tipularia discolor*, respectively. Debeljak *et al*. (2002) induced tubers of *Pterostylis sanguinea in vitro* when seedlings were cultured with jasmonic acid alone or in combination with sucrose. Unfortunately, not all terrestrial orchids

form storage organs. Although these methods do improve seedling survival, more studies are needed to monitor long term seedling survival after reintroduction. Survival is dependent on mycorrhizal recruitment, soil composition, timing of reintroduction, storage organ size, seedling size, and environmental conditions.

12. SEED STORAGE

Many orchid species are now imperiled with habitat degradation and loss, threatening species survival. Although habitat preservation and *in situ* conservation are often the desired approach for species conservation, *ex situ* conservation is essential for long term storage of genetic germplasm (Pritchard and *Seaton* 1993). Methods for conserving material *ex situ* include species cultivation, clonal propagation, and long term pollen/seed storage with seed storage being the most attractive option (Pritchard and *Seaton* 1993).

Orchid seeds are classified as orthodox meaning seed longevity can be enhanced by reducing their moisture content from 20% to 5% (relative humidity of 11%), and by lowering storage temperatures to 0°C (Pritchard *et al.* 1999). Viability can be retained 5-20 years at refrigerator temperatures depending on the species being stored (Shoushtari *et al.* 1994). Pritchard and *Seaton* (1993) reported high seed viability after six years at -20°C and -196°C, but other species' seeds are sensitive to desiccation at -20°C and below. Seed moisture content above 10.4% can lead to severe viability loss (Pritchard 1984). For example, *Eulophia alta* seeds with a moisture content of 23% reduced germination 20% after 2 months storage at 2°C (Pritchard 1984). If storing seed at low temperatures, finding the correct desiccant is important. Silica gel reduced the seed moisture content of *Cattleya aurantiaca* to 2.2% after one week; however, calcium chloride maintained the optimum seed moisture content of 5.6% (*Seaton* and Hailes 1989).

Pritchard (1984) first reported the successful storage of orchid seeds in liquid nitrogen (LN) at -196°C (=cryopreservation). Since Pritchard published the first cryopreservation study on orchid seeds others have reported successful cryopreservation of many orchid species. Tropical, temperate, epiphytic, and terrestrial orchid seeds have all been successfully cryopreserved by directly plunging seeds contained in cryovials into LN with little to no loss of viability (Nikishina *et al*. 2001a, 2001b; Popova *et al*. 2003; Nikishina *et al*. 2007).

A vitrification method has also been used to pretreat seeds before placing them into LN. In this process, a large portion of the freezable water is dehydrated at a non-freezing temperature (Hirano *et al.* 2005b). This ensures that cell damaging ice crystals do not form upon freezing (Vendrame *et al*. 2007). Often called osmoprotection, tissues are treated with a 2 *M* glycerol and 0.4 *M* sucrose cryoprotectant solution usually at room temperature. Vitrification eliminates the need for slow /careful freezing, and also allows seeds to be safely placed into LN (Thammasiri 2000). The vitrification method often leads to increased germination, decreased seedling damage, and increased seedling survival (Ishikawa *et al.* 1997; Thammasiri 2000; Hirano *et al*. 2005a, 2005b). Zygotic embryos and seeds of *Bletilla striata* have been cryopreserved through vitrification (Ishikawa *et al*. 1997; Hirano *et al*. 2005a). Immature seeds of *Ponerorchis graminifolia* var. *suzukiana* were also cryopreserved through vitrification (Hirano *et al*. 2005b). Vendrame *et al*. (2007) reported higher germination of several *Dendrobium* hybrids when seeds were exposed to vitrification at 0°C compared to 27°C before cryopreservation. Care must be taken to find the optimum vitrification temperature and exposure times to increase survivorship and germination (Vendrame *et al*. 2007). In all studies, embryo and seedling morphology were normal with no morphological abnormalities.

As habitat is lost or degraded, not only are orchids imperiled but also their mycobionts. For conservation purposes, preserving the fungi that form this symbiosis with orchids is important. Techniques for storing orchid seeds and their mycobionts simultaneously have been explored. Wood *et al*. (2000) stored seeds of two European terrestrial orchids along with their mycobionts in sodium alginate beads before placing them in LN. Encapsulated seeds were pretreated with several sucrose concentrations from 0 to 1 *M*, but optimum seed germination and fungal hyphae growth occurred with 0.75 *M* sucrose. Encapsulated seeds and fungi were stored at 16°C, -20°C, -70°C, or LN for 0, 3, and 30 days. When stored at -196°C, 100% germination occurred at all time periods (Wood *et al*. 2000). Batty *et al*. (2001) reported on the cryopreservation of several Australian terrestrial orchid seeds and their mycobionts. Along with the seeds, a 3 mm^2 fungal agar cube was placed in the cryovial. Following 12 months in storage at 22°C, 4°C, -18°C, and LN, seed germination was higher after storage in LN for three species. When seeds and fungi were stored together in LN, germination was higher for four species than using LN seed and non-LN fungi, non-LN seed and LN fungi, or non-LN seed and fungi.

Cryopreservation offers a promising method for long term storage of orchid germplasm. Since cryopreservation seems to appear species specific in relation to pretreatment methods, more species need to be included in cryopreservation studies. This area of research will be extremely important as more orchids are faced with extinction, and long term storage may be the only way to preserve germplasm for long periods of time.

13. CONCLUSION

An abundance of research is currently ongoing that involves orchid seeds and various aspects of *in vitro* germination. This review demonstrates that parameters for orchid seed germination appear to be species specific. In order to optimize germination, researchers should attempt to mimic the natural growing conditions experienced by each species, whether those conditions are photoperiod or temperature. To thoroughly study *in vitro* seed germination, both asymbiotic and symbiotic germination are necessary. Asymbiotic germination is an excellent technique to study biotic and abiotic factors of orchid seed biology, while symbiotic germination provides a way to investigate the physiological correct mechanism of orchid seed germination. Although great advances are being made, a lack of understanding in orchid seed physiology, ecology, and whole plant ecology remains. Future research must examine dormancy mechanisms and the techniques to overcome these dormancies. Also long-term germplasm storage should be investigated more thoroughly. Finally, if conservation is an ultimate goal for seed germination experiments, reintroduction or field transplant methods must be examined further. Few published articles exist on field transplanting orchid seedlings or plants, and limited knowledge about field survival still exists. Many orchid species are threatened with extinction from land conversion, as well habitat mismanagement. The goal for most orchid conservation research should be to manage existing orchid populations to prevent population and species loss. Researching orchid seed germination, physiology, and ecology is a key step in the recovery of at risk populations and species.

REFERENCES

Anderson A (1996) The reintroduction of *Platanthera ciliaris* in Canada. In: Allen C (Ed) *North American Native Terrestrial Orchids Propagation and Production*, North American Native Terrestrial Orchid Conference, Germantown, Maryland, pp 63-71

Arditti J (1967) Factors affecting the germination of orchid seed. *The Botanical Review* **33**, 1-97

Arditti J (1982) Orchid seed germination and seedling culture: A manual. In: Arditti J (Ed) *Orchid Biology: Reviews and Perspectives* (Vol II), Cornell University Press, Ithaca, NY, pp 245-370

Arditti J, Michaud JD, Oliva AP (1981) Seed germination of North American orchids. I. native California and related species of *Calypso*, *Epipactis*, *Goodyera*, *Piperia*, and *Platanthera*. *Botanical Gazette* **142**, 442-453

Arditti J, Ghani AKA (2000) Numerical and physical properties of orchid seeds and their biological implications. *New Phytologist* **145**, 367-421

Ballard WW (1990) Further notes on *Cypripedium* germination. In: Sawyers CE (Ed) *North American Native Terrestrial Orchid Propagation and Production*, Brandywine Conservancy, Chadds Ford, Pennsylvania, pp 87-89

Baskin CC, Baskin JM (2001) *Seeds Ecology, Biogeography, and Evolution of Dormancy and Germination*, Academic Press, San Diego, CA, 665 pp

Baskin CC, Baskin JM (2004a) Determining dormancy-breaking and germination requirements from the fewest seeds. In: Guerrant EO, Havens K, Maunder M (Eds) *Ex Situ Plant Conservation: Supporting Species Survival in the Wild*, Island Press, Washington, DC, pp 162-179

Baskin JM, Baskin CC (2004b) A classification system for seed dormancy. *Seed Science Research* **14**, 1-16

Baskin CC, Thompson K, Baskin JM (2006) Mistakes in germination ecology and how to avoid them. *Seed Science Research* **16**, 165-168

Batty AL, Dixon KW, Brundrett M, Sivathisamparam K (2001) Long-term storage of mycorrhizal fungi and seed as a tool for the conservation of endangered Western Australian terrestrial orchids. *Australian Journal of Botany* **49**, 619-628

Batty Al, Brundrett MC, Dixon KW, Sivasithamparam K (2006a) New methods to improve symbiotic propagation of temperate terrestrial orchid seedlings from axenic culture to soil. *Australian Journal of Botany* **54**, 367-674

Batty Al, Brundrett MC, Dixon KW, Sivasithamparam K (2006b) *In situ* seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. *Australian Journal of Botany* **54**, 375-381

Bernard N (1899) Sur la germination du *Neottia nidus* avis. *Comptes Rendus de l'Académie des Sciences Paris* **128**, 1253-1255

Bewley JD, Black M (1994) *Seeds: Physiology of Development and Germination*, Plenum Press, New York, 462 pp

Beyrle HF, Smith SE, Peterson RL, Franco CMM (1995) Colonization of *Orchis morio* protocorms by a mycorrhizal fungus: effects of nitrogen nutrition and glyphosate in modifying the responses. *Canadian Journal of Botany* **73**, 1128-1140

Brundrett MC, Scade A, Batty AL, Dixon KW, Sivasithamparam K (2003) Development of *in situ* and *ex situ* seed baiting techniques to detect mycorrhizal fungi from terrestrial orchid habitats. *Mycological Research* **107**, 1210-1220

Burgeff H (1909) *Die Wurzelpilze der Orchideen, ihre Kultur und ihre Leben in der Pflanze*, G. Fischer Verlag, Jena, 202 pp

Cardenas EC, Wang TT (1998) The effect of micronutrients and GA on the growth of *Phalaenopsis* seedlings *in vitro*. *Subtropical Plant Science* **50**, 45-48

Chu CC, Mudge KW (1994) Effects of prechilling and liquid suspension culture on seed germination of the yellow lady's slipper orchid (*Cypripedium calceolus* var. *pubescens*). *Lindleyana* **9**, 153-159

Coke JL (1990) Aseptic germination and growth of some terrestrial orchids. In: Sawyers CE (Ed) *North American Native Terrestrial Orchids Propagation and Production*, Brandywine Conservancy, Chadds Ford, Pennsylvania, pp 90-91

Crafts CB, Miller CO (1974) Detection and identification of cytokinins produced by mycorrhizal fungi. *Plant Physiology* **54**, 586-588

Curtis JT (1947) Studies on the nitrogen nutrition of orchid embryos. I. complex nitrogen sources. *American Orchid Society Bulletin* **16**, 654-660

Curtis JT, Spoerl E (1948) Studies on the nitrogen nutrition of orchid embryos. II. comparative utilization of nitrate and ammonium nitrogen. *American Orchid Society Bulletin* **17**, 111-114

Debeljak N, Regvar M, Dixon KW, Sivasithamparam K (2002) Induction of tuberisation in vitro with jasmonic acid and sucrose in an Australian terrestrial orchid, *Pterostylis sanguinea*. *Plant Growth Regulation* **36**, 235-260

de Pauw MA, Remphrey WR (1993) *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media, and cold treatment. *Canadian Journal of Botany* **71**, 879-885

de Pauw MA, Remphrey WR, Palmer CE (1995) The cytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum*. *Annals of Botany* **75**, 267-275

Dijk E, Olff H (1994) Effects of nitrogen, phosphorus, and potassium fertilization on field performance of *Dactylorhiza majalis*. *Acta Botanic Neerlandica* **43**, 383-392

Dijk E, Eck N (1995a) Axenic *in vitro* nitrogen and phosphorus responses of some Dutch marsh orchids. *New Phytologist* **131**, 353-359

Dijk E, Eck N (1995b) Ammonium toxicity and nitrate response of axenically grown *Dactylorhiza incarnata* seedlings. *New Phytologist* **131**, 361-367

Dimalla GG, van Staden J (1977) The effect of temperature on the germination and endogenous cytokinin and gibberellin levels of pecan nuts. *Zeitschrift für Pflanzenphysiologie* **82**, 274-280

Dixon K (1987) Raising terrestrial orchids from seed. In: Harris WK (Ed) *Modern Orchid Growing for Pleasure and Profit*, Orchid Club of South Australia, Adelaide, South Australia, pp 47-100

Dressler RL (1993) *Phylogeny and Classification of the Orchid Family*, Press Syndicate of the University of Cambridge, Melbourne, Australia, 330 pp

Ernst R, Arditti J (1990) Carbohydrate physiology of orchid seedlings. III. Hydrolysis of maltooligosaccharides by *Phalaenopsis* seedlings. *American Journal of Botany* **77**, 188-195

Ernst R, Bjornsen JE, Arditii J (1992) Effects of ethephon, its nonethylene-generating analog ethylphosphoric acid, and phosphoric acid in aseptic culture of orchid seedlings. *American Journal of Botany* **79**, 275-278

Feurtado JA, Ambrose SJ, Cutler AJ, Ross ARS, Abrams SR, Kermode AR (2004) Dormancy termination of western pine (*Pinus monticola* Dougl. Ex D. Don) seeds is associated with changes in abscisic acid metabolism. *Planta* **218**, 630-639

Fukai S, Fujiwara K, Okamoto O, Hasegawa A, Goi M (1997) Effects of red and blue light on germination and protocorm growth of *Calanthe* Satsuma. *Lindleyana* **12**, 169-171

Hirano T, Godo T, Mii M, Ishikawa K (2005a) Cryopreservation of immature seeds of *Bletilla striata* by vitrification. *Plant Cell Reports* **25**, 534-539

Hirano T, Ishikawa K, Mii M (2005b) Cryopreservation of immature seeds of *Ponerorchis graminifolia* var. *suzukiana* by vitrification. *CryoLetters* 26, 139-146

Hufford KM, Mazer SJ (2003) Plant ecotypes: genetic differentiation in the age of ecological restoration. *Trends in Ecology and Evolution* **18**, 147-155

Ishikawa K, Harata K, Mii M, Sakai A, Yoshimatsu K, Shimomura K (1997) Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. *Plant Cell Reports* **16**, 754-757

Johnson TR, Stewart SL, Dutra D, Kane ME, Richardson L (2007) Asymbiotic and symbiotic seed germination of Eulophia alta (Orchidaceae)-preliminary evidence for the symbiotic culture advantage. *Plant Cell, Tissue and Organ Culture* **90**, 313-323

Kauth PJ (2005) *In vitro* seed germination and seedling development of *Calopogon tuberosus* and *Sacoila lanceolata* var. *lanceolata*: two Florida native terrestrial orchids. MS thesis, Environmental Horticulture Department, University of Florida, 91 pp

Kauth PJ, Vendrame WA, Kane ME (2006) *In vitro* seed culture and seedlings development of *Calopogon tuberosus*. *Plant Cell, Tissue and Organ Culture* **85**, 91-102

Kermode AG (2005) Role of abscisic acid in seed dormancy. *Journal of Plant Growth Regulation* **24**, 319-344

Kettenring KM, Gardner G, Galatowitsch SM (2006) Effect of light on seed germination of eight wetland *Carex* species. *Annals of Botany* **98**, 869-874

Knudson L (1922) Nonsymbiotic germination of orchid seed. *Botanical Gazette* **73**, 1-25

Knudson L (1946) A new nutrient solution for the germination of orchid seed. *American Orchid Society Bulletin* **15**, 214-217

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Review* **15**, 281-307

Lakon G (1949) The topographical tetrazolium method for determining the germinating capacity of seeds. *Plant Physiology* **24**, 389-394

Lauzer D, St-Arnaud M, Barabé D (1994) Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). *Lindleyana* **9**, 197-204

Lee YI, Lee N, Yeung EC, Chung MC (2005) Embryo development of *Cypripedium formosanum* in relation to seed germination *in vitro*. *Journal of the American Society for Horticultural Science* **130**, 747-753

Lee YI, Yeung EC, Lee N, Chung MC (2006) Embryo development in the lady's slipper orchid, *Paphiopedilum delenatii*, with emphasis on the ultrastructure of the suspensor. *Annals of Botany* **98**, 1311-1319

Lee YI, Lu CF, Chung MC, Yeung EC, Lee N (2007) developmental changes in endogenous abscisic acid concentrations and asymbiotic seed germination of a terrestrial orchid, *Calanthe tricarinata* Lindl. *Journal of the American Society for Horticultural Science* **132**, 246-252

Leon RG, Owen MDK (2003) Regulation of weed seed dormancy through light and temperature interactions. *Weed Science* **51**, 752-758

Liberman M (1979) Biosynthesis and action of ethylene. *Annual Review of Plant Physiology* **30**, 533-591

Linden B (1992) Two new methods for pretreatment of seeds of Northern orchids to improve germination in axenic culture. *Annales Botanici Fennici* **29**, 305-313

Lo SF, Nalawade SM, Kuo CL, Chen CL, Tsay HS (2004) Asymbiotic germination of immature seeds, plantlet development and *ex vitro* establishment of plants of *Dendrobium tosaense* Makino – a medicinally important orchid. *In Vitro Cellular and Developmental Biology – Plant* **40**, 528-535

Majerowicz N, Kerbauy GB, Nievola CC, Suzuki RM (2000) Growth and nitrogen metabolism of *Catasetum fimbriatum* (Orchidaceae) grown with different nitrogen sources.

Environmental and Experimental Botany **44**, 195-206
Malmgren S (1993) Large-scale asymbiotic propagation of *Cypripedium calceolus*-plant physiology from a surgeon's point of view. *Botanic Gardens Micropropagation News* **1**, 59-63
Malmgren S (1996) Orchid propagation: theory and practice. In: Allen C (Ed) *North American Native Terrestrial Orchids Propagation and Production*, North American Native Terrestrial Orchid Conference, Germantown, Maryland, pp 63-71
Manning JC, van Staden J (1987) The development and mobilization of seed reserves in some African orchids. *Australian Journey of Botany* **35**, 343-353
McKinley T, Camper ND (1997) Action spectra of *in vitro* asymbiotic germination of *Goodyera repens* var. *ophioides*. *Lindleyana* **12**, 30-33
Miyoshi K, Mii M (1988) Ultrasonic treatment for enhancing seed germination of terrestrial orchid, *Calanthe discolor*, in asymbiotic culture. *Scientia Horticulturae* **35**, 127-130
Miyoshi K, Mii M (1995) Phytohormone pretreatment for the enhancement of seed germination and protocorm formation by the terrestrial orchid, *Calanthe discolor* (Orchidaceae), in asymbiotic culture. *Scientia Horticulturae* **63**, 263-267
Miyoshi K, Mii M (1998) Stimulatory effects of sodium and calcium hypochlorite, pre-chilling and cytokinins on the germination of *Cypripedium macranthos* seed *in vitro*. *Physiologia Plantarum* **102**, 481-486
Molvray M, Kores PJ (1995) Character analysis of the testa in Spiranthoideae and Orchidoideae, with special reference to the Diurideae (Orchidaceae). *American Journal of Botany* **82**, 1443-1454
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497
Nikishina TV, Popov AS, Kolomeitseva GL, Golovkin BN (2001a) Effect of cryoconservation on seed germination of rare tropical orchids. *Russian Journal of Plant Physiology* **48**, 810-815
Nikishina TV, Popov AS, Kolomeitseva GL, Golovkin BN (2001b) Cryopreservation of seeds of some tropical orchids. *Doklady Biochemistry and Biophysics* **378**, 231-233
Nikishina TV, Popova EV, Vakhrameeva MG, Varlygina TI, Kolomeitseva GL, Burov AV, Popovich EA, Shirokov AI, Shumilov VY, Popov AS (2007) Cryopreservation of seeds and protocorms of rare temperate orchids. *Russian Journal of Plant Physiology* **54**, 121-127
Oddie RLA, Dixon KW, McComb JA (1994) Influence of substrate on asymbiotic and symbiotic *in vitro* germination and seedling growth of two Australian terrestrial orchids. *Lindleyana* **9**, 183-189
Otero JT, Ackerman JD, Bayman P (2004) Differences in mycorrhizal preferences between two tropical orchids. *Molecular Ecology* **13**, 2393-2404
Pedroza-Manrique J, Fernandez-Lizarazo C, Suarez-Silva A (2005) Evaluation of the effect of three growth regulators in the germination of *Comperettia falcata* seeds under in vitro conditions. *In Vitro Cellular and Developmental Biology – Plant* **41**, 838-843
Popova EV, Nikishina TV, Kolomeitseva GL, Popov AS (2003) The effect of seed cryopreservation on the development of protocorms by the hybrid orchid *Bratonia*. Russian *Journal of Plant Physiology* **50**, 672-677
Pritchard HW (1984) Liquid Nitrogen preservation of terrestrial and epiphytic orchid seed. *CryoLetters* **5**, 295-300
Pritchard HW, Seaton PT (1993) Orchid seed storage: historical perspective, current status, and future prospects for long-term conservation. *Selbyana* **14**, 89-104
Pritchard HW, Poynter ALC, Seaton PT (1999) Interspecific variation in orchid seed longevity in relation to ultra-dry storage and cryopreservation. *Lindleyana* **14**, 92-101
Prusch J, Schardt A, Schill R (2000) Adaptations of an orchid seed to water uptake and storage. *Plant Systematics and Evolution* **220**, 69-75
Raghavan V (1964) Effects of certain organic nitrogen compounds on growth in vitro of seedlings of *Cattleya*. *Botanical Gazette* **125**, 260-267
Raghavan V, Torrey JG (1964) Inorganic nitrogen nutrition of the seedling of the orchid, *Cattleya*. *American Journal of Botany* **51**, 264-274
Rasmussen HN (1992) Seed dormancy patterns in *Epipactis palustris* (Orchidaceae): Requirements for germination and establishment of mycorrhiza. *Physiologia Plantarum* **86**, 161-167
Rasmussen HN (1995) *Terrestrial Orchids from Seed to Mycotrophic Plant*, Cambridge University Press, Cambridge, UK, 456 pp
Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. *Plant and Soil* **244**, 149-163
Rasmussen HN, Anderson TF, Johansen B (1990a) Light stimulation and darkness requirements for the symbiotic germination of *Dactylorhiza majalis* (Orchidaceae) *in vitro*. *Physiologia Plantarum* **79**, 226-230
Rasmussen HN, Anderson TF, Johansen B (1990b) Temperature sensitivity of *in vitro* germination and seedling development of *Dactylorhiza majalis* (Orchidaceae) with and without a mycorrhizal fungus. *Plant, Cell and Environment* **13**, 171-177
Rasmussen HN, Rasmussen FN (1991) Climactic and seasonal regulation of seed plant establishment in *Dactylorhiza majalis* inferred from symbiotic experiments *in vitro*. *Lindleyana* **5**, 221-227
Rasmussen HN, Whigham DF (1993) Seed ecology of dust seed *in situ*: a new study technique and its application in terrestrial orchids. *American Journal of Botany* **80**, 1374-1378
Scade A, Brundrett MC, Batty AL, Dixon KW, Sivasithamparam K (2006) Survival of transplanted terrestrial orchid seedlings in urban bushland habitats with high or low weed cover. *Australian Journal of Botany* **54**, 383-389
Seaton PT, Hailes NSJ (1989) Effect of temperature and moisture content on the viability of *Cattleya aurantiaca* seed. In: Pritchard HW (Ed) *Modern Methods in Orchid Conservation*, Cambridge University Press, Cambridge, UK, pp 17-29
Sharma J, Zettler LW, Van Sambeek JW, Ellersieck MR, Starbuck CJ (2003) Symbiotic seed germination and mycorrhizae of federally threatened *Platanthera praeclara* (Orchidaceae). *American Midland Naturalist* **149**, 104-120
Shimura H, Koda Y (2005) Enhanced symbiotic seed germination of *Cypripedium macranthos* var. *rebunense* following inoculation after cold treatment. *Physiologia Plantarum* **123**, 281-287
Shimura H, Matsuura M, Takada N, Koda Y (2007) An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae). *Phytochemistry* **68**, 1442-1447
Shoushtari BD, Heydari R, Johnson GL, Arditti J (1994) Germination and viability staining of orchid seeds following prolonged storage. *Lindleyana* **9**, 77-84
Spoerl E, Curtis JT (1948) Studies of the nitrogen nutrition of orchid embryos. III. amino acid nitrogen. *American Orchid Society Bulletin* **17**, 307-312
St-Arnaud M, Lauzer D, Barabé D (1992) *In vitro* germination and early growth of seedlings of *Cypripedium acaule* (Orchidaceae). *Lindleyana* **7**, 22-27
Steele WK (1996) Large scale seedling production of North American *Cypripedium* species. In: Allen C (Ed) *Proceedings of the North American Native Terrestrial Orchid-Propagation and Production Conference*, North American Native Terrestrial Orchid Conference, Germantown, Maryland, pp 11-26
Stenberg ML, Kane ME (1998) *In vitro* seed germination and greenhouse cultivation of *Encyclia boothiana* var. *erythronioides*, an endangered Florida orchid. *Lindleyana* **13**, 101-112
Stewart SL, Kane ME (2006a) Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell, Tissue and Organ Culture* **86**, 147-158
Stewart SL, Kane ME (2006b) Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell, Tissue and Organ Culture* **86**, 159-167
Stewart SL, Kane ME (2007) Symbiotic germination and evidence for *in vitro* mycobiont specificity in *Spiranthes brevilabris* (Orchidaceae), and its implications for species-level conservation. *In Vitro Cellular and Developmental Biology – Plant* **43**, 178-186
Stewart SL, Zettler LW (2002) Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinqueseta*, *H. macroceratitis*) from Florida. *Aquatic Botany* **72**, 25-35
Stoutamire WP (1964) Seeds and seedlings of native orchids. *Michigan Botanist* **3**, 107-119
Stoutamire WP (1974) Terrestrial orchid seedlings. In: Withner CL (Ed) *The Orchids: Scientific Studies*, John Wiley and Sons, New York, pp 101-128
Szendrak E, Read PE (2000) *In vitro* propagation and anatomical studies of temperate orchid species (Orchidaceae). *Acta Horticulturae* **520**, 75-80
Taiz L, Zeiger E (1998) *Plant Physiology*, Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts, 700 pp
Takahashi K, Ogiwara I, Hakoda N (2000) Seed germination of *Habenaria* (*Pecteilis*) *radiata* (Orchidaceae: Orchideae) *in vitro*. *Lindleyana* **15**, 59-63
Thammasiri K (2000) Cryopreservation of seeds of a Thai orchid (*Doritis pulcherrima* Lindl.) by vitrification. *CryoLetters* **21**, 237-244
Tissue DT, Skillman JB, McDonald EP, Strain BR (1995) Photosynthesis and carbon allocation in *Tipularia discolor* (Orchidaceae), a wintergreen understory herb. *American Journal of Botany* **85**, 1249-1256
Tomita M, Tomita M (1997) Effects of culture media and cold treatment on germination in asymbiotic culture of *Cypripedium macranthos* and *Cypripedium japonicum*. *Lindleyana* **12**, 208-213
Vacin EF, Went FW (1949) Some pH changes in nutrient solutions. *Botanical Gazette* **110**, 605-613
van der Kinderen G (1987) Abscisic acid in terrestrial orchid seeds: a possible impact on their germination. *Lindleyana* **2**, 84-87
van Waes JM, Debergh PC (1986a) Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. *Physiologia Plantarum* **66**, 435-442
van Waes JM, Debergh PC (1986b) *In vitro* germination of some Western European orchids. *Physiologia Plantarum* **67**, 253-261
van Staden J, Webb DP, Wareing PF (1972) The effect of stratification on endogenous cytokinin levels in seeds of *Acer saccharum*. *Planta* **104**, 110-114
Vendrame WA, Carvalho VS, Dias JMM (2007) *In vitro* germination and seedling development of cryopreserved Dendrobium hybrid mature seeds. *Scientia Horticulturae* **114**, 188-193
Walck JL, Hidayati SN (2005) Differences in light and temperature responses determine autumn versus spring germination for seeds of *Schoenoliron croceum*. *Canadian Journal of Botany* **82**, 1429-1437
Wood CB, Pritchard HW, Miller AP (2000) Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation-dehydration is dependent on moisture content and

storage temperature. *CryoLetters* **21**, 125-136
Yamazaki J, Miyoshi K (2006) *In vitro* asymbiotic germination of immature seed and formation of protocorms by *Cephalanthera falcata* (Orchidacaea). *Annals of Botany* **98**, 1197-1206
Yeung EC, Zee SY, Ye XL (1996) Embryology of *Cymbidium sinense*: embryo development. *Annals of Botany* **78**, 105-110
Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water uptake by means of mycotrophy. *Plant Science* **156**, 145-150
Zettler LW (1996) Symbiotic seed germination of terrestrial orchid in North America during the last decade: a progress report. In: Allen C (Ed) *North American Native Terrestrial Orchids Propagation and Production*, North American Native Terrestrial Orchid Conference, Germantown, Maryland, pp 43-53
Zettler LW (1997a) Orchid-fungal symbiosis and its value in conservation. *McIlvainea* **13**, 40-45
Zettler LW (1997b) Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. *Selbyana* **18**, 188-194
Zettler LW (2001) Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. In: Higgins WE, Walsh BW (Eds) *Orchid Conservation Proceedings: An International Conference on Orchid Conservation*, Selby Botanical Gardens Press, Sarasota, FL, pp 51-57
Zettler LW, Hofer CJ (1997) Sensitivity of *Spiranthes odarata* seeds to light during *in vitro* symbiotic seed germination. *Lindleyana* **12**, 26-29
Zettler LW, Hofer CJ (1998) Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination, and its ecological implications. *Environmental and Experimental Botany* **39**, 189-195
Zettler LW, McInnis TM (1992) Propagation of *Platanthera integrilabia* (Correll) Luer, and endangered terrestrial orchid, through symbiotic seed germination. *Lindleyana* **7**, 154-161
Zettler LW, McInnis TM (1994) Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (*Platanthera integrilabia*). *Plant Science* **102**, 133-138
Zettler LW, Burkhead JC, Marshall JA (1999) Use of a mycorrhizal fungus from *Epidendrum conopseum* to germinate seed of *Encyclia tampensis in vitro*. *Lindleyana* **14**, 102-105
Zettler LW, Sharma J, Rasmussen FN (2003) Mycorrhizal diversity. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (Eds) *Orchid Conservation*, Natural History Publications (Borneo) Sdn. Bhd., Kota Kinabalu, Sabah, Malaysia, pp 205-226
Zettler LW, Poulter SB, McDonald KI, Stewart SL (2007) Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. *HortScience* **42**, 135-139
Zettler LW, Stewart SL, Bowles ML, Jacobs KA (2001) Mycorrhizal fungi and cold-assisted symbiotic germination of the federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley. *American Midland Naturalist* **145**, 168-175
Zettler LW, Perlman S, Dennis DJ, Hopkins SE, Poulter SB (2005) Symbiotic germination of a federally endangered Hawaiian endemic, *Platanthera holochila* (Orchidaceae), using a mycobiont from Florida: a conservation dilemma. *Selbyana* **26**, 269-276
Zettler LW, Piskin KA, Stewart SL, Hartsock JJ, Bowles ML, Bell TJ (2005b) Protocorm mycobionts of the federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindley, and a technique to prompt leaf elongation in seedlings. *Studies in Mycology* **53**, 1-10
Ziegler AR, Sheehan TJ, Poole RT (1967) Influence of various media and photoperiod on growth and amino acid content of orchid seedlings. *American Orchid Society Bulletin* **36**, 195-202